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Charles P. Landrum

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Copland et al.

Serial No.: 09/418,095

Filed: October 14, 1999

For: THIAZOLIDINEDIONES IN COMBINATION WITH OTHER THERAPEUTIC AGENTS FOR TUMOR THERAPY

Group Art Unit: 1636

Examiner: Nguyen, Q.

Atty. Dkt. No.: UTSG:239

APPEAL BRIEF

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APPEAL BRIEF

BOX AF
Commissioner of Patents
Washington, D.C. 20231

Commissioner:

Applicants hereby submit an original and two copies of this Appeal Brief to the Board of Patent Appeals and Interferences in response to the final Office Action dated July 01, 2002. The fee for filing this Appeal Brief is $320, and is attached hereto. This Brief was due on January 7, 2003 based on the receipt date of the Notice of Appeal. A request for a one month extension is filed concurrently herewith and results in a due date of February 7, 2003.

If the check is inadvertently omitted, or should any additional fees under 37 C.F.R. §§ 1.16 to 1.21 be required for any reason relating to the enclosed material, or should an overpayment be included herein, the Commissioner is authorized to deduct or credit said fees from or to Fulbright & Jaworski L.L.P. Account No.: 50-1212/UTSG:239.

I. STATUS OF THE CLAIMS

Appellants' amendment dated August 29, 2002 was not entered, as stated in the Advisory Action mailed on September 23, 2002. Claims 1-46 are pending in the application.

II. STATUS OF AMENDMENTS

Appellants are filing an amendment, concurrent with this brief, to address a minor inconsistency within claims, typographical errors and to address objections to the specification. A copy of the Amendment is provided as Exhibit A. A copy of the pending claims if the amendment is entered is provided in Exhibit B. A copy of the pending claims if the amendment is not entered is provided in Exhibit C.

III. REAL PARTY IN INTEREST

The real party in interest is The Board of Reagents, the University of Texas System in Austin, Texas.

IV. RELATED APPEALS AND INTERFERENCES

There are no related appeals or interferences.

V. SUMMARY OF THE INVENTION

The invention is directed generally to the use of thiazolidinedione compounds, including troglitizone, in combination with other cancer therapies for the treatment of cancer.

In various embodiments, methods for inhibiting the growth of a cancer cell includes contacting the cancer cell with a thiazolidinedione compound and contacting the cancer cell with a chemotherapeutic drug or irradiating the cancer cell with X-ray irradiation, UV-irradiation, γ-irradiation, or microwaves, in amounts effective to inhibit the growth of the cancer cell.

In certain embodiments the thiazolidinedione compound is a troglitazone, a pioglitazone or a rosiglitazone.

In some embodiments the cancer cell is a mammalian cancer cell. In particular embodiments the cancer cell is a human cancer cell.

In various embodiments, contacting the cancer cell occurs *in vitro*. In other embodiments contacting the cancer cell occurs *in vivo*.

In some embodiments, the cancer cell is selected from a group consisting of a bladder, blood, bone, bone marrow, brain, breast, colon, esophagus, gastrointestine, head, kidney, liver, lung, nasopharynx, neck, ovary, prostate, skin, stomach, and uterus cell.

In various embodiments, the cancer cell expresses PPAR-γ.

In still other embodiments, the cancer cell is a bone cancer cell, an osteosarcoma cell, a precursor to osteosarcoma, an ovarian cancer cell, or a renal cancer cell.

In certain embodiments, the cancer cell is contacted with a chemotherapeutic drug. In some embodiments, the chemotherapeutic drug includes an alkylating agent, a mitotic inhibitor, an antibiotic, a nitrosurea, an antimetabolite, a corticosteroid hormone, or other antineoplastic agent. In certain embodiments, the chemotherapeutic drug comprises an alklyating agent, a mitotic inhibitor, an antibiotic, a nitrosourea, an antimetabolite, a corticosteroid hormone or an antineoplastic agent.

In various embodiments, the thiazolidinedione compound is contacted with a cancer cell by administering the thiazolidinedione regionally, endoscopically, intravenously, intralesionally, percutaneously, subcutaneously, intraperitoneally, intratracheally, intramuscularly, or by perfusion.

In some embodiments, the thiazolidinedione and the chemotherapeutic drug are suitably dispersed in a pharmacologically acceptable formulation. In certain embodiments, the

thiazolidinedione compound is contacted with the cancer cell at the same time as contact with the chemotherapeutic agent.

In certain embodiments, the cancer cell is a tumor cell in a tumor.

In other embodiments, the method includes resecting the tumor.

In various embodiments, the cancer cell is irradiated with X-ray irradiation, UV-irradiation, -irradiation, or microwaves. In certain embodiments, the thiazolidinedione compound is contacted with the cancer cell at the same time as irradiation.

In some embodiments, the methods include contacting the cancer cell with a therapeutic polynucleotide selected from the group consisting of a Dp gene, p21, p16, p27, E2F, Rb, APC, DC, NF-1, NF-2, WT-1, MEN-I, MEN-II, BRCA1, VHL, FCC, MCC, *ras*, *myc*, *neu*, *raf*, *erb*, *src*, *fms*, *jun*, *trk*, *ret*, *gsp*, *hst*, *bcl*, *abl*, Bax, Bcl-X_S and E1A; wherein the therapeutic polynucleotide is expressed in the cancer cell.

Various embodiments of the invention include methods for treating cancer in a patient comprising administering to the patient troglitazone and a chemotherapeutic drug in an amount effective to produce a therapeutic benefit.

Other embodiments of the invention include methods for inhibiting the cell cycle progression of a mammalian cancer cell comprising contacting the cell with an amount of troglitazone and a chemotherapeutic drug effective to inhibit the cell cycle progression of the cell.

In still other embodiments of the invention, the method of treating cancer in a patient includes administering to the patient a therapeutically effective amount of troglitazone and a chemotherapeutic drug.

In yet other embodiments, the methods include treating microscopic residual cancer comprising the steps of identifying a patient having a resectable tumor, resecting said tumor, and contacting the tumor bed with a therapeutically effective amount of troglitazone and a chemotherapeutic drug.

Some embodiments of the invention include a method for treating a subject having a tumor comprising the steps of surgically revealing said tumor, and contacting said tumor with a therapeutically effective amount of troglitazone and a chemotherapeutic drug.

Other embodiments of the invention include methods for treating a subject having a tumor comprising the step of perfusing said tumor, over an extended period of time, with a therapeutically effective amount of troglitazone and a chemotherapeutic drug. In other embodiments, the thiazolidinedione and the chemotherapeutic agent are combined in a therapeutic formulation.

Various embodiments include a method for inhibiting the growth of a cancer cell comprising contacting the cancer cell with a composition comprising troglitazone and contacting the cancer cell with a chemotherapeutic agent or irradiating the cancer cell, in amounts effective to inhibit growth of the cancer cell. In certain embodiments, the cancer cell is contacted with a chemotherapeutic agent. In some embodiments, the composition comprises troglitazone and a chemotherapeutic agent. In various embodiments, the cancer cell is a bone cancer cell, an osteosarcoma cell, an ovarian cancer cell, or a renal cancer cell.

Support for the invention as summarized may be found in the specification at least on pages 6 to 11.

VI. ISSUES ON APPEAL

A. Is claim 32 enabled under of 35 U.S.C. §112?

B. Are claims 1-8, 16, 28, 33-35 and 40-41 anticipated under 35 U.S.C. §102(e) by US Patent 5,814,647 ("Urban")(Exhibit D) as evidenced by US Patents 5,736,129 ("Medenica")(Exhibit E); 6,090,407 ("Knight")(Exhibit F); and 5,747,469 ("Roth")(Exhibit G)?

C. Are claims 1-31 and 33-46 obvious under 35 U.S.C. §103(a) over Tontonoz *et al.* (Exhibit H) in view of U.S. Patents 5,814,647 ("Urban"), 5,736,129 ("Medenica"), 6,090,407 ("Knight"), and 5,747,469 ("Roth")?

VII. GROUPING OF THE CLAIMS

Claims 1-8, 16, 28, 33-35 and 40-41 stand or fall together with respect to rejection under 35 U.S.C. §102(e).

Claims 1-31 and 33-46 do not stand or fall together with respect to rejection under 35 U.S.C. §103(a). In particular claims 8 and 35 are distinct from all other claims because they have additional limitations and additional arguments can be set forth regarding these claims; claims 16-24 are distinct from all other claims because they have additional limitations and additional arguments can be set forth regarding these claims; claim 25 is distinct from all other claims because this claim has additional limitations and additional arguments can be set forth regarding this claim; 27 is distinct from all other claims because this claim has additional limitations and additional arguments can be set forth regarding this claim; claims 29-31 are distinct from all other claims because these claims have additional limitations and additional arguments can be set forth regarding these claims; claim 33 is distinct from all other claims because this claim has additional limitations and additional arguments can be set forth regarding this claim; claim 36 is distinct from all other claims because this claim has additional limitations

and additional arguments can be set forth regarding this claim; claim 37 is distinct from all other claims because this claim has additional limitations and additional arguments can be set forth regarding this claim; claim 38 is distinct from all other claims because this claim has additional limitations and additional arguments can be set forth regarding this claim; and claim 39 is distinct from all other claims because this claim has additional limitations and additional arguments can be set forth regarding this claim. Thus, each group of claims is differentiated by additional limitations that are non-obvious over the cited references and each group of claims defines patentably distinct subject matter.

Claim 32 stands alone with respect to the rejection under 35 U.S.C. §112, first paragraph.

VIII. SUMMARY OF ARGUMENT

A. Claim 32 satisfies the requirements of 35 U.S.C. §112.

As explained in detail below, the present rejection of claim 32 under 35 U.S.C. §112 is based on unpredictability in the art, such that undue experimentation would be required to practice the invention. The arguments in support of the rejection are based on over-interpretation of the art as it relates to the claimed invention and improper application of broad generalizations of the field of gene therapy to the claimed invention.

B. Claims 1-8, 16, 28, 33-35 and 40-41 are patentable under 35 U.S.C. §102(e).

As explained in detail below, the present rejection of claims 1-8, 16, 28, 33-35 and 40-41 under 35 U.S.C. §102(e) is based on an improper combination of references to provide purported enablement for the primary reference. The Appellants have advanced that the lack of an enabling disclosure in a primary reference cannot be remedied by the combination of references. The primary reference itself must be enabling and the combination of references for a rejection

under 35 U.S.C. §102 must be cited to ***prove*** that the primary reference contains an "enabled disclosure" not to ***provide*** enablement for the primary reference. Thus, enablement must be found in the primary reference and not in the combination of references.

C. Claims 1-31 and 33-46 are patentable under 35 U.S.C. §103(a).

As explained in detail below, the present rejection of claims 1-31 and 33-46 under 35 U.S.C. §103(a) is based on a faulty analysis of the primary reference and fails to establish a *prima facie* case of obviousness. At most, the references cited disclose that one skilled in the art might find it "obvious-to-try" the claimed invention. In addition, the primary reference, Tontonoz *et al.*, actually teaches away from the invention. Further, the Examiner fails to consider unexpected results provided by the Appellants.

IX. ARGUMENT

A. Claim 32 Is Enabled Under 35 U.S.C. § 112, First Paragraph

The final Office Action dated July 1, 2002 ("Action") rejected claim 32 under 35 U.S.C. § 112, first paragraph, as lacking enablement. The Action states that the claim encompasses gene therapy for the treatment of cancer *in vivo*, and due to the unpredictability in the art, it would require undue experimentation to practice the invention. Appellants respectfully traverse this rejection.

Appellants submit that Claim 32 stands alone and is distinct from all other pending claims due to the fact that the limitations of the claim are (1) not found in any single prior art reference or (2) the combination of these limitations are not found in a combination of prior art reference in a manner in which the claimed invention as a whole would be obvious to one of ordinary skill in the art. The fact that no prior art has been cited against claim 32, when all other

pending claims are subject to a rejection based on prior art evinces the separate patentability of claim 32. Thus, if claim 32 is found to be enabled then claim 32 is allowable over pending 35 U.S.C. §102(e) and §103(a) rejections.

Satisfaction of the enablement requirement is not precluded by the necessity of some experimentation. *See Atlas Powder Co. v. E.I. duPont De Nemours & Co.,* 750 F.2d 1569, 1576, 224 U.S.P.Q. 409 (Fed. Cir. 1984). "The specification must teach those skilled in the art how to make and use the full scope of the claimed invention without 'undue experimentation.'" MPEP 2164.08 (citing *In re Wright*, 999 F.2d 1557, 1561, 27 U.S.P.Q. 1510, 1513 (Fed. Cir. 1993)). The invention of claim 32 encompasses a method for inhibiting the growth of a cancer cell comprising contacting the cancer cell with a thiazolidinedione compound; contacting the cancer cell with a chemotherapeutic drug or irradiating the cancer cell with X-ray, UV-irradiation, γ-irradiation, or microwaves, in amounts effective to inhibit the growth of the cancer cell; and contacting the cancer cell with a therapeutic polynucleotide, wherein the therapeutic polynucleotide is expressed in the cancer cell. Methods of contacting a cell with a polynucleotide such that the polynucleotide is expressed in the cell were well known in the art at the time of the present invention. Furthermore, proof of efficacy in clinical trials involving humans is not a requirement for patentability. *See In re Brana,* 51 F.3d 1560 (Fed. Cir. 1995). *See also Scott v. Finney*, 34 F.3d 1058, 1063, 32 U.S.P.Q.2d 1115, 1120 (Fed. Cir. 1994) ("Title 35 does not demand that such human testing occur within the confines of Patent and Trademark (PTO) proceedings.").

The Action cites Dang, *Clin. Cancer Res.*, 5:471-474 (1999) (hereinafter Dang, Exhibit I), for the proposition that the field of gene therapy will need further advancement to make it a reality. The Action lists several factors identified by Dang that are known to limit the

effectiveness of gene therapy including: the lack of optimal vectors, the lack of stable *in vivo* transgene expression, the adverse host immune response, and the lack of efficient gene delivery to target tissues. Appellants respectfully point out, however, that Dang focuses on clinical issues, which are above and beyond the standards for patentability. *See In re Krimmel*, 292 F.2d 948, 954 (C.C.P.A. 1961) ("There is nothing in the patent statute or any other statutes . . .which gives the Patent Office the right or the duty to require an applicant to prove that compounds or other materials which he is claiming, and which he has stated are useful for 'pharmaceutical applications,' are safe, effective, and reliable for use with humans."). These generalities in no way preclude or render unpredictable cancer therapy using genetic material.

Although some obstacles have been encountered in certain gene therapy applications, there have also been many successes in the field. For example, Dang reports that there are approximately 300 approved gene therapy trials (page 471, first paragraph). In addition, Dang discusses studies using HIV-based vectors that have shown "no evidence of brisk immune responses" to the vectors (paragraph bridging page 471 and 472). Even in studies where the host developed an immune response to the vector, the transgene was still expressed (Dang, page 473, paragraph bridging column 1 and 2). Furthermore, clinical trail data suggest that E1A-liposome gene therapy is a feasible cancer treatment (Dang, page 474, column 1, first full paragraph).

The Action also cites Eck & Wilson, *Gene-based Therapy* (1996) (hereinafter Eck, Exhibit J) for additional factors that may influence the effects sought to be achieved through gene therapy. The Action specifically identifies the unpredictability in levels of transgene expression, the duration of the expression, and the *in vivo* therapeutic effects. Again, Eck addresses the clinical effectiveness of gene therapy as a whole. It is important, however, to recognize the differences between using gene therapy to treat cancer as compared to inherited

disorders. As Eck points out, gene therapy for acquired disorders, such as cancer, is mechanistically more flexible than gene therapy for inherited disorders (page 78, column 2, last partial paragraph). For example, the duration of expression of the transgene in cancer therapy is less of an issue than it is for inherited disorders. In fact, in the treatment of a malignancy, the long-term expression of the therapeutic protein could potentially have deleterious consequences (Eck, page 82, column 1, first full paragraph).

Eck actually re-enforces the Appellants' assertion that methods of delivering therapeutic polynucleotides to cells were well known in the art at the time the invention was made. Numerous DNA delivery strategies are described in the article including viral vectors such as retrovirus, adenovirus, adeno-associated virus, and herpes simplex virus-1 (Eck, page 83-89). Several nonviral delivery strategies are also discussed, including liposomes, uncomplexed DNA, DNA-coated gold particles, and DNA-protein conjugates (Eck, page 90-92). Likewise, the other articles cited in the Action, Miller & Vile, *FASEB*, 9:190-99 (1995)(Exhibit K); Deonarian, *Exp. Opin. Ther. Patents*, 8:53-69 (1998)(Exhibit L); and Verma & Somia, *Nature*, 389:239-242 (1997)(Exhibit M), also describe numerous methods for the transfer and expression of polynucleotides. Applicants contend that the level of skill in the art at the time of the present invention was such that the guidance provided in the present disclosure would enable one skilled in the art to make and use the claimed invention. As such, the enablement rejection of claim 32 is improper and should be withdrawn.

B. Claims 1-8, 16-23, 28, 30, 33-35 and 40-41 are not Anticipated by the Cited References

The Final Office Action mailed on July 1, 2002 ("Action") rejected claims 1-8, 16-23, 28, 30, 33-35 and 40-41 under 35 U.S.C. § 102(e) as being anticipated by Urban *et al.* (U.S. Patent

No. 5,814,647) ("Urban") as evidenced by Medenica *et al.* (U.S. Patent No. 5,736,129) ("Medenica"), Knight *et al.* (U.S. Patent No. 6,090,407) ("Knight") and Roth *et al.* (U.S. Patent No. 5,747,469) ("Roth"). The Action states that Urban teaches that troglitazone and related thiazolidinedione compounds can be used in the treatment of climacteric and cancer. The Examiner also argues that Urban ***suggests*** the use of troglitazone therapy in conjunction with chemotherapeutic agents, radiation, or surgery. Although, Urban ***does not teach*** specifically the chemotherapeutic drugs or types of radiation used, the Examiner argues that the types of chemotherapeutic drugs and the types of radiation used in the treatment of cancer are well known in the art as evidenced by Medenica, Knight, and Roth. Appellants respectfully traverse this rejection.

For a prior art disclosure to anticipate an applicant's invention, the reference must contain an "enabling disclosure." MPEP §2121.01 (quoting *In re Hoeksema*, 399 F.2d 269, 158 U.S.P.Q. 596 (C.C.P.A. 1968)). In addition, the amount of guidance needed to enable the invention is inversely related to the predictability in the art. MPEP §2164.03 (citing *In re Fischer*, 427 F.2d 833, 839, 166 U.S.P.Q. 18, 24 (C.C.P.A. 1970)). Appellants submit that Urban does not contain an enabling disclosure for methods of treatment as claimed and that the use of secondary references is improper and cannot remedy the non-enabling disclosure of Urban.

The Appellants' invention is directed towards a method for inhibiting the growth of a cancer cell by treating the cancer with a ***combination*** of a thiazolidinedione compound with other therapies. In particular, the methods of the presently claimed invention are directed towards a method for inhibiting the growth of a cancer cell comprising contacting the cancer cell with a thiazolidinedione compound and contacting the cancer cell with a chemotherapeutic drug

or irradiating the cancer cell with x-ray irradiation, UV-irradiation, γ-irradiation, or microwaves, in amounts effective to inhibit the growth of the cancer cell. Prior to the Appellants' disclosure it was not ***known*** whether the use of thiazolidinedione therapy in ***combination*** with other chemotherapeutic agents or radiation would inhibit the growth of a cancer cell, in particular if inhibition would be less than, equal to, or more than each therapy alone. Based on the art cited the success of the combination therapy could not have been predicted and one of skill in the art would not come to the conclusion that the art cited would work as claimed in the present application.

The disclosure by Urban of the use of troglitazone in combination with chemotherapeutic drugs or radiation, which is provided in two sentences, was merely a prophetic statement with no basis for combination therapy provided in the disclosure, experimental or otherwise. Due to the lack of predictability in the art, such a disclosure would not enable one of ordinary skill in the art to make and use the invention. It is not enough for the Urban reference to state a goal with words like that of the claimed invention without an accompanying enabling disclosure. Urban does not show that the invention claimed in the present application would work. In fact, Urban merely states, "Use of Troglitazone therapy in conjunction with other chemotherapeutic agents, radiation, or surgery ***may*** in many cases be the preferred mode of treatment. Troglitazone treatment therefore, would inhibit the growth of the cancer so that other therapies ***may*** be added, thereby increasing the likelihood of curing the patient." This clause makes clear that Urban does ***not*** teach that administration of a thiazolidinedione compound in combination with a chemotherapeutic drug or irradiation in amounts effective to inhibit the growth of the cancer cell.

Furthermore, as stated in the final Office Action on page 11: "...Urban does not teach specifically the types of chemotherapeutic drugs and the types of radiation used in combination

with the troglitazone therapy..." The courts have ruled that the "tossing out the mere germ of an idea does not constitute an enabling disclosure" and that it is "the specification, not knowledge in the art, that must supply the novel aspects of an invention in order to constitute adequate enablement." *Genentech, Inc. v. Novo Nordisk A/S*, 42 USPQ 2d 1001. Urban fails to enable the use of troglitazone in combination with chemotherapeutic drugs or radiation. The statements of Urban, see the proceeding paragraph, may suggest using troglitazone with other therapies, but these statements do not teach a method, taken alone or in combination with Medenica, Knight, or Roth, for inhibiting the growth of a cancer cell by treating the cancer with a ***combination*** of a thiazolidinedione compound with other therapies. Appellants note that there is no description of combination therapy in Urban and no working or prophetic example is provided. At most, combination therapy, as described in Urban, was a mere germ of an idea that is not supported by the Urban disclosure. Thus, the use of Urban as an anticipatory reference is improper due to the fact that Urban, taken alone or in combination with secondary references, ***does not teach*** one of ordinary skill ***methods of treatment*** using troglitazone in combination with chemotherapeutics or irradiation.

The deficiency of the Urban disclosure can not be remedied by using secondary references. Multiple references may be used to a) prove that the primary reference contains an enabling disclosure (*In re Samour*, 571 F.2d 559, 197 USPQ 1 (CCPA 1978) and *In re Donohue*, 766 F.2d 531 226 USPQ 619 (Fed. Cir. 1985)), b) explain the meaning of a term used in the primary reference (*In re Baxter Travenol Labs*, 952 F.2d 388, 21 USPQ2d 1281 (Fed. Cir. 1991)), or c) show that a characteristic not disclosed in the reference is inherent (*Continental Can Co. USA v Monsanto Co.*, 948 F2d 1264, 1268; 20 USPQ2d 1746, 1749 (Fed. Cir. 1991)) (as described in MPEP 2131.01). The Examiner cites the secondary references to provide, not

prove, enablement of the Urban disclosure. The Medenica, Knight, and Roth references can not be used to ***provide*** enablement, *i.e.*, enablement must be found in the anticipating reference not in the supporting references. Proving enablement is distinct in that proving is showing that enablement is inherent in the anticipating reference, which is not the case for the Urban reference as described below. Further, the references fail to provide enablement for treating cancer by using a combination of *thiazolidinedione* with other chemotherapeutic agents or radiation. In fact none of the secondary references describe or mention the use of thiazolidinedione in combination with other chemotherapeutic agents or radiation. Applicants note, for example, that the teaching in Urban, alone or in combination with Medenica, Knight, and Roth, does not provide any teaching that would have lead one of ordinary skill in the art to predict that the combination therapy taught in the instant specification would have resulted in a reduction of 5-FU dose by a factor of 100 (see at least page 57 lines 25-28 and FIG. 5). In fact, one of ordinary skill would be unable to predict if a therapy using thiazolidinedione in combination with other chemotherapeutic agents or radiation would inhibit the growth of a cancer cell, in particular whether the inhibition would be less than, equal to, or more than each therapy alone. Accordingly, for the above reasons, Appellants contend that claims 1-8, 16-23, 28, 30, 33-35 and 40-41 are not anticipated under 35 U.S.C. § 102(e) by Urban *et al.* in light of Medenica, Knight, and Roth.

C. Claims 1-31 and 33-46 are Non-obvious over the Cited References

The Action rejects claims 1-31 and 33-46 under 35 U.S.C. § 103(a) as being obvious over Tontonoz *et al.* (*Proc. Natl. Acad. Sci.* 94:237-241) ("Tontonoz") in view of Urban *et al.* (U.S. Patent No. 5,814,647), Medenica *et al.* (U.S. Patent No. 5,736,129), Knight *et al.* (U.S. Patent No. 6,090,407), and Roth *et al.* (U.S. Patent No. 5,747,469). The Action alleges that it would

have been obvious to a person of ordinary skill in the art at the time the invention was made to modify the method disclosed by Tontonoz by combining the use of a thiazolidinedione compound in conjunction with other chemotherapeutic agents to inhibit the growth of liposarcoma cells or mesenchymal tumor cells, or tumor cells expressing PPARγ as taught by Urban. Appellants respectfully traverse this rejection.

In order to establish a *prima facie* case of obviousness, the teaching of the claimed combination and the reasonable expectation of success must both be found in the prior art. MPEP §2143 (citing *In re Vaeck*, 947 F.2d 488, 20 U.S.P.Q.2d 1438 (Fed. Cir. 1991)). Tontonoz does not teach or suggest that the references be combined and in fact does not teach a combination therapy. Tontonoz actually teaches *away* from combining thiazolidinedione therapy with chemotherapeutic drugs or radiation by recommending the use of thiazolidinedione compounds as an ***alternative*** to conventional chemotherapy. Tontonoz states on p. 237, in the paragraph spanning the left and right columns that: "Induction of terminal differentiation represents a promising ***alternative*** to conventional chemotherapy for certain malignancies." (emphasis added) and on page 241, second full paragraph "Our results suggest that the thiazolidinedione class of antidiabetic drugs and RXR-specific retinoids may be useful as a nontoxic ***alternative*** to conventional chemotherapy for the treatment of disseminated or locally advanced liposarcoma." (emphasis added). The term "alternative" as defined in Webster's New Twentieth Century Dictionary, Unabridged, defines "alternative" as "that which may be chosen or omitted as one of two things, so that if one is taken, the other must be left." (Exhibit N) Thus, the Tontonoz reference teaches away from the combination of thiazolidinedione and conventional chemotherapy by providing thiazolidinedione therapy as an alternative to conventional chemotherapy.

Furthermore, Tontonoz as a whole describes a combination of two compounds that act on the same molecular entity, a PPARγ/RXRα heterodimeric steroid hormone receptor. The teaching of Tontonoz is essentially a single therapy consisting of the activation a PPARγ/RXRα heterodimeric steroid hormone receptor. At most, Tontonoz describes the effect of thiazolidinediones and other PPARγ/RXRα ligands in the treatment of cancer. Tontonoz does not contain any suggestion or motivation to combine its teachings with other references beyond PPARγ/RXRα ligands for the activation of a particular steroid hormone receptor.

Urban is said to disclose the use of troglitazone therapy in conjunction with other chemotherapeutic agents or radiation; however, Urban does not teach the use of any specific chemotherapeutic drugs or types of radiation. Moreover, the statement disclosing the use of troglitazone in combination with chemotherapeutic drugs or radiation is a mere suggestion with no teaching of a method using troglitazone therapy in conjunction with other chemotherapeutic agents or radiation. One of ordinary skill in the art, in light of the teaching Tontonoz in view of Urban, would not be able to make and use the claimed invention, or have a reasonable expectation that such a method would succeed. As discussed in the argument against the anticipation rejection above, the Urban reference is not enabled for a "method for inhibiting the growth of a cancer cell comprising contacting the cancer cell with a thiazolidinedione compound and contacting the cancer cell with a chemotherapeutic drug or irradiating the cancer cell with x-ray irradiation, UV-irradiation, γ-irradiation, or microwaves, in amounts effective to inhibit the growth of the cancer cell."

Furthermore, none of the other cited references remedies this defect. Applicants note, for example, that the teaching in Urban, alone or in combination with Medenica, Knight, and Roth, does not provide any teaching that would have lead one of ordinary skill in the art to predict that

the combination therapy taught in the instant specification would have resulted in a reduction of 5-FU dose by a factor of 100 (see at least page 57 lines 25-28 and FIG. 5). A fact that the Examiner has failed to consider. Therefore, in the cited references, there is no reasonable expectation of success in practicing the claimed invention. The only expectation of success in this instance is derived from the application itself, which is not permitted (*Hodosh v Block Drug Co. Inc.*, 786 F.2d 1136, 1143 n.5, 229 USPQ 182, 187 n.5 (Fed. Cir. 1986); MPEP 2141) Accordingly, there is no *prima facie* case of obviousness.

At most, the references disclose that one skilled in the art might find it "obvious-to-try" the claimed invention. An "obvious-to-try" situation exists when a general disclosure piques the scientist's curiosity, "such that further investigation might be done as a result of the disclosure, but the disclosure itself does not contain sufficient teaching of how to obtain the desired result, or that the claimed result would be obtained if certain directions were pursued." *In re Eli Lilly & Co.*, 902 F.2d 943, 945, 14 U.S.P.Q.2d 1741, 1743 (Fed. Cir. 1990). The Federal Circuit has consistently held that "obvious to try" is not to be equated with obviousness under 35 U.S.C. §103. *Gillette Co. v. S.C. Johnson & Son, Inc.*, 919 F.2d 720, 725, 16 U.S.P.Q.2d 1923, 1928 (Fed. Cir. 1990). This rejection is inappropriate and should be withdrawn.

In regard to claims 1-31 and 33-46 not standing or falling together with respect to rejection under 35 U.S.C. §103(a) the following reasoning is provided with incorporation of the argumentation presented above. In order to establish a *prima facie* case of obviousness there must be, in the references taken as a whole, a teaching of the claimed combination and a reasonable expectation of success. Furthermore, the prior art references when combined must teach or suggest ***all claim limitations***. MPEP §2143 (citing *In re Rouffet*, 149 F.3d 1350, 1357 47 USPQ2d 1453, 1457-58 (Fed. Cir. 1998); *In re Merck & Co., Inc.* 800 F.2d 1091, 231 USPQ

375 (Fed. Cir. 1986); *In re Vaeck*, 947 F.2d 488, 20 U.S.P.Q.2d 1438 (Fed. Cir. 1991) and *In re Royka*, 490 F.2d 981, 180 USPQ 580 (CCPA 1974)).

Claims 8 and 35 are distinct from all other claims due to the fact that these claims further comprise the inhibition of cancer cells *in vivo*. The references, purportedly supporting a 35 U.S.C. §103(a), taken as a whole, do not teach all the limitations provided in claim 8 or 35. Thus, there are additional arguments for the withdrawal of the 35 U.S.C. §103(a) rejections of claims 8 and 35. Accordingly, claims 8 and 35 do not stand or fall with claims 1-7, 9-31, 33-34, and 36-46.

Claims 16-24 are distinct from all other claims due to the fact that these claims further comprise administration of particular chemotherapeutic agents or classes of chemotherapeutic agents in combination with thiazolidinedione. The references, purportedly supporting a 35 U.S.C. §103(a), taken as a whole, do not teach all limitations provided in claim 16-24 nor do the references provide a reasonable expectation for the success of the invention as claimed in 16-24. Thus, there are additional arguments for the withdrawal of the 35 U.S.C. §103(a) rejections of the claims. Accordingly, claims 8 and 35 do not stand or fall with claims 1-7, 9-31, 33-34, and 36-46.

Claim 25 is distinct from all other claims due to the fact that this claim further comprises administration of a thiazolidinedione compound by various methods of administration. The references, purportedly supporting a 35 U.S.C. §103(a), taken as a whole do not suggest or motivate one to combine the references, provide a reasonable expectation for success, or teach all limitations provided in claim 25 for a combination treatment using a thiazolidinedione compound administered regionally, endoscopically, intravenously, intralesionally, percutaneously, subcutaneously, intraperitoneally, intratracheally, intramuscularly, or by perfusion. Thus, there

are additional arguments for the withdrawal of the 35 U.S.C. §103(a) rejection of the claim. Accordingly, claim 25 does not stand or fall with claims 1-24, 26-31, and 33-46.

Claim 27 is distinct from all other claims due to the fact that this claim is directed to the inhibition of cancer cells by administration of a thiazolidinedione compound and a chemotherapeutic agent simultaneously. The references, purportedly supporting a 35 U.S.C. §103(a), taken as a whole do not suggest or motivate one to combine the references, provide a reasonable expectation for success, or teach all limitations provided in claim 27. Thus, there are additional arguments for the withdrawal of the 35 U.S.C. §103(a) rejection of the claim. Accordingly, claim 27 does not stand or fall with claims 1-26, 28-31, and 33-46.

Claims 29-31 are distinct from all other claims due to the fact that these claims further comprise administration of a thiazolidinedione compound and a chemotherapeutic drug or irradiating the cancer cell with X-ray irradiation, UV-irradiation, γ-irradiation, or microwaves, in amounts effective to inhibit the growth of a tumor cell, and further comprising resecting the tumor and contacting the tumor at the same time as irradiation. The references, purportedly supporting a 35 U.S.C. §103(a), taken as a whole do not suggest or motivate one to combine the references, provide a reasonable expectation for success, or teach all limitations provided in claims 29-31. Thus, there are additional arguments for the withdrawal of the 35 U.S.C. §103(a) rejections of the claims. Accordingly, claims 29-31 do not stand or fall with claims 1-28 and 33-46.

Claim 33 is distinct from all other claims due to the fact that this claim comprises administering to the patient troglitazone and a chemotherapeutic drug in an amount effective to produce a therapeutic benefit. The references, purportedly supporting a 35 U.S.C. §103(a), taken as a whole do not suggest or motivate one to combine the references, provide a reasonable

expectation for success, or teach all limitations provided in claim 33. Thus, there are additional arguments for the withdrawal of the 35 U.S.C. §103(a) rejections of the claims. Accordingly, claim 33 does not stand or fall with claims 1-31 and 34-46.

Claim 36 is distinct from all other claims due to the fact that this claim comprises the steps of identifying a patient having a resectable tumor, resecting said tumor, and contacting the tumor bed with a therapeutically effective amount of troglitazone and a chemotherapeutic drug. The references, purportedly supporting a 35 U.S.C. §103(a), taken as a whole do not suggest or motivate one to combine the references, provide a reasonable expectation for success, or teach all limitations provided in claim 36. Thus, there are additional arguments for the withdrawal of the 35 U.S.C. §103(a) rejections of the claims. Accordingly, claim 36 does not stand or fall with claims 1-31 and 33-35, and 37-46.

Claim 37 is distinct from all other claims due to the fact that this claim comprises the steps of surgically revealing a tumor, and contacting the tumor with a therapeutically effective amount of troglitazone and a chemotherapeutic drug. The references, purportedly supporting a 35 U.S.C. §103(a), taken as a whole do not suggest or motivate one to combine the references, provide a reasonable expectation for success, or teach all limitations provided in claim 37. Thus, there are additional arguments for the withdrawal of the 35 U.S.C. §103(a) rejections of the claims. Accordingly, claim 37 does not stand or fall with claims 1-31 and 33-36, and 38-46.

Claim 38 is distinct from all other claims due to the fact that this claim comprises the step of perfusing a tumor, over an extended period of time, with a therapeutically effective amount of troglitazone and a chemotherapeutic drug. The references, purportedly supporting a 35 U.S.C. §103(a), taken as a whole do not suggest or motivate one to combine the references, provide a reasonable expectation for success, or teach all limitations provided in claim 38. Thus, there are

additional arguments for the withdrawal of the 35 U.S.C. §103(a) rejections of the claims. Accordingly, claim 38 does not stand or fall with claims 1-31 and 33-37, and 39-46.

Claim 39 is distinct from all other claims due to the fact that this claim requires that the thiazolidinedione and the chemotherapeutic agent are combined in a therapeutic formulation. The references, purportedly supporting a 35 U.S.C. §103(a), taken as a whole do not suggest or motivate one to combine the references, provide a reasonable expectation for success, or teach all limitations provided in claim 39. Thus, there are additional arguments for the withdrawal of the 35 U.S.C. §103(a) rejections of the claims. Accordingly, claim 39 does not stand or fall with claims 1-31 and 33-38, and 40-46.

X. CONCLUSION

In light of the foregoing, Appellants respectfully submit that the claims on appeal satisfy 35 U.S.C. §112, are not anticipated under 35 U.S.C. §102(e) by the cited art, and are not rendered obvious under 35 U.S.C. §103(a) by the cited art. Reconsideration and withdrawal of the rejection is respectfully requested.

Please date stamp and return the enclosed postcard to evidence receipt of this document.

Respectfully submitted,

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Reg. No. 46,855
Agent for Applicant

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Date: February 7, 2003

CERTIFICATE OF MAILING
37 C.F.R. 1.8

I hereby certify that this correspondence is being deposited with the U.S. Postal Service with sufficient postage as First Class Mail in an envelope addressed to: Commissioner for Patents, Washington, DC 20231, on the date below:

February 7, 2003
Date

Charles P. Landrum

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
John A. Copland, III
Slavisa Gasic
Randall J. Urban
Melvyn Soloff

Serial No.: 09/418,095

Filed: October 14, 1999

For: THIAZOLIDINEDIONES IN COMBINATION WITH OTHER THERAPEUTIC AGENTS FOR TUMOR THERAPY

Group Art Unit: 1646

Examiner: Nguyen, Q.

Atty. Dkt. No.: UTSG:239/GNS

AMENDMENT UNDER 37 C.F.R. § 1.116

BOX AF
Commissioner for Patents
Washington, D.C. 20231

Commissioner:

Applicants respectfully request that the following amendment be entered in the captioned patent application in accordance with 37 C.F.R. § 1.116. Applicants submit the foregoing amendment to place the case in even better condition for allowance or appeal.

It is believed that no fee is due; however, should any fees under 37 C.F.R. §§ 1.16 to 1.21 be required for any reason relating to this document, the Commissioner is authorized to deduct said fees from Fulbright & Jaworski Account No.: 50-1212/UTSG:239.

Reconsideration of the application in view of the following amendment and remarks is respectfully requested.

AMENDMENT

Please make the following amendments.

In the Specification:

On page 15 please replace the phrase "FIG. 17" with "FIG. 17A-17C".

On page 15 please replace the phrase "FIG. 18" with "FIG. 18A-18B".

In the Claims:

Please amend claims to read as follows.

18. (Amended) The method of claim 17, wherein the chemotherapeutic drug comprises an alkylating agent.

34. (Amended) A method for inhibiting the cell cycle progression of a mammalian cancer cell comprising contacting the cell with an amount of troglitazone and a chemotherapeutic drug effective to inhibit the cell cycle progression of the cell.

REMARKS

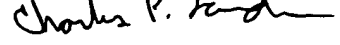
Claims 1-46 are pending. Claims 18 and 34 are amended herein. The amendment to claim 18 is to correct a typographical error in the word "alkylating". The amendment to claim 34

is to make the scope of this claim consistent with the scope of the other claims by reciting "and a chemotherapeutic drug."

Support for the amended claims can be found in the Specification and in the originally filed claims that form part of this application's written description. Applicants contend that no new matter has been added. A copy of the claim amendments can be found in Appendix A. A copy of the pending claims including the amendments made herein is provided in Appendix B.

This amendment is filed concurrently with an Appeal Brief. The Examiner is invited to contact the undersigned agent at (512) 536-5674 with any questions, comments or suggestions relating to the referenced patent application.

Respectfully submitted,



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APPENDIX A:
VERSION OF CLAIM AMENDMENTS MARKED TO SHOW CHANGES

In the claims:

18. (Amended) The method of claim 17, wherein the chemotherapeutic drug comprises an alkylating [alklyating] agent.

34. A method for inhibiting the cell cycle progression of a mammalian cancer cell comprising contacting the cell with an amount of troglitazone and a chemotherapeutic drug effective to inhibit the cell cycle progression of the cell.

APPENDIX B:
PENDING CLAIMS (UNOFFICIAL)

1. A method for inhibiting the growth of a cancer cell comprising
   (i) contacting the cancer cell with a thiazolidinedione compound; and
   (ii) contacting the cancer cell with a chemotherapeutic drug or irradiating the cancer cell with X-ray irradiation, UV-irradiation, γ-irradiation, or microwaves, in amounts effective to inhibit the growth of the cancer cell.

2. The method of claim 1, wherein the thiazolidinedione compound is a troglitazone.

3. The method of claim 1, wherein the thiazolidinedione compound is a pioglitazone.

4. The method of claim 1, wherein the thiazolidinedione compound is a rosiglitazone.

5. The method of claim 1, wherein the cancer cell is a mammalian cancer cell.

6. The method of claim 5, wherein the cancer cell is a human cancer cell.

7. The method of claim 1, wherein the contacting occurs *in vitro*.

8. The method of claim 1, wherein the contacting occurs *in vivo*.

9. The method of claim 1, wherein the cancer cell is selected from a group consisting of a bladder, blood, bone, bone marrow, brain, breast, colon, esophagus, gastrointestine, head, kidney, liver, lung, nasopharynx, neck, ovary, prostate, skin, stomach, and uterus cell.

10. The method of claim 9, wherein the cancer cell expresses PPAR-γ.

11. The method of claim 9, wherein the cancer cell is a bone cancer cell.

12. The method of claim 11, wherein the bone cancer cell is an osteosarcoma cell.

13. The method of claim 11, wherein the cancer cell is a precursor to osteosarcoma.

14. The method of claim 9, wherein the cancer cell is an ovarian cancer cell.

15. The method of claim 9, wherein the cancer cell is a renal cancer cell.

16. The method of claim 1, wherein the cancer cell is contacted with a chemotherapeutic drug.

17. The method of claim 16, wherein the chemotherapeutic drug comprises an alkylating agent, mitotic inhibitor, antibiotic, nitrosurea, antimetabolite, corticosteroid hormone, or other antineoplastic agent.

18. The method of claim 17, wherein the chemotherapeutic drug comprises an alkylating agent.

19. The method of claim 17, wherein the chemotherapeutic drug comprises a mitotic inhibitor.

20. The method of claim 17, wherein the chemotherapeutic drug comprises an antibiotic.

21. The method of claim 17, wherein the chemotherapeutic drug comprises a nitrosourea.

22. The method of claim 17, wherein the chemotherapeutic drug comprises an antimetabolite.

23. The method of claim 17, wherein the chemotherapeutic drug comprises a corticosteroid hormone.

24. The method of claim 17, wherein the chemotherapeutic drug comprises an antineoplastic agent.

25. The method of claim 1, wherein the thiazolidinedione compound is contacted with a cancer cell by administering the thiazolidinedione regionally, endoscopically, intravenously, intralesionally, percutaneously, subcutaneously, intraperitoneally, intratracheally, intramuscularly, or by perfusion.

26. The method of claim 17, wherein the thiazolidinedione and the chemotherapeutic drug are suitably dispersed in a pharmacologically acceptable formulation.

27. The method of claim 1, wherein the thiazolidinedione compound is contacted with the cancer cell at the same time as contact with the chemotherapeutic agent.

28. The method of claim 1, wherein the cancer cell is a tumor cell in a tumor.

29. The method of claim 28, further comprising resecting the tumor.

30. The method of claim 28, wherein the cancer cell is irradiated with X-ray irradiation, UV-irradiation, -irradiation, or microwaves.

31. The method of claim 30, wherein the thiazolidinedione compound is contacted with the cancer cell at the same time as irradiation.

32. The method of claim 25, further comprising contacting the cancer cell with a therapeutic polynucleotide selected from the group consisting of a Dp gene, p21, p16, p27, E2F, Rb, APC, DC, NF-1, NF-2, WT-1, MEN-I, MEN-II, BRCA1, VHL, FCC, MCC, *ras*, *myc*, *neu*, *raf*, *erb*, *src*, *fms*, *jun*, *trk*, *ret*, *gsp*, *hst*, *bcl*, *abl*, Bax, Bcl-X_S and E1A; wherein the therapeutic polynucleotide is expressed in the cancer cell.

33. A method for treating cancer in a patient comprising administering to the patient troglitazone and a chemotherapeutic drug in an amount effective to produce a therapeutic benefit.

34. A method for inhibiting the cell cycle progression of a mammalian cancer cell comprising contacting the cell with an amount of troglitazone and a chemotherapeutic drug effective to inhibit the cell cycle progression of the cell.

35. A method of treating cancer in a patient comprising administering to the patient a therapeutically effective amount of troglitazone and a chemotherapeutic drug.

36. A method for treating microscopic residual cancer comprising the steps of:
(i) identifying a patient having a resectable tumor;
(ii) resecting said tumor; and
(iii) contacting the tumor bed with a therapeutically effective amount of troglitazone and a chemotherapeutic drug.

37. A method for treating a subject having a tumor comprising the steps of:
(i) surgically revealing said tumor; and
(ii) contacting said tumor with a therapeutically effective amount of troglitazone and a chemotherapeutic drug.

38. A method for treating a subject having a tumor comprising the step of perfusing said tumor, over an extended period of time, with a therapeutically effective amount of troglitazone and a chemotherapeutic drug.

39. The method of claim 27, wherein the thiazolidinedione and the chemotherapeutic agent are combined in a therapeutic formulation.

40. A method for inhibiting the growth of a cancer cell comprising i) contacting the cancer cell with a composition comprising troglitazone and ii) contacting the cancer cell with a chemotherapeutic agent or irradiating the cancer cell, in amounts effective to inhibit growth of the cancer cell.

41. The method of claim 40, wherein the cancer cell is contacted with a chemotherapeutic agent.

42. The method of claim 41, wherein the composition comprises troglitazone and a chemotherapeutic agent.

43. The method of claim 40, wherein the cancer cell is a bone cancer cell.

44. The method of claim 43, wherein the bone cancer cell is an osteosarcoma cell.

45. The method of claim 40, wherein the cancer cell is an ovarian cancer cell.

46. The method of claim 40, wherein the cancer cell is a renal cancer cell.

EXHIBIT B

COPY OF PENDING CLAIMS IF AMENDMENT IS ENTERED

1. A method for inhibiting the growth of a cancer cell comprising:
   (i) contacting the cancer cell with a thiazolidinedione compound; and
   (ii) contacting the cancer cell with a chemotherapeutic drug or irradiating the cancer cell with X-ray irradiation, UV-irradiation, γ-irradiation, or microwaves, in amounts effective to inhibit the growth of the cancer cell.

2. The method of claim 1, wherein the thiazolidinedione compound is a troglitazone.

3. The method of claim 1, wherein the thiazolidinedione compound is a pioglitazone.

4. The method of claim 1, wherein the thiazolidinedione compound is a rosiglitazone.

5. The method of claim 1, wherein the cancer cell is a mammalian cancer cell.

6. The method of claim 5, wherein the cancer cell is a human cancer cell.

7. The method of claim 1, wherein the contacting occurs *in vitro*.

8. The method of claim 1, wherein the contacting occurs *in vivo*.

9. The method of claim 1, wherein the cancer cell is selected from a group consisting of a bladder, blood, bone, bone marrow, brain, breast, colon, esophagus, gastrointestine, head, kidney, liver, lung, nasopharynx, neck, ovary, prostate, skin, stomach, and uterus cell.

10. The method of claim 9, wherein the cancer cell expresses PPAR-γ.

11. The method of claim 9, wherein the cancer cell is a bone cancer cell.

12. The method of claim 11, wherein the bone cancer cell is an osteosarcoma cell.

13. The method of claim 11, wherein the cancer cell is a precursor to osteosarcoma.

14. The method of claim 9, wherein the cancer cell is an ovarian cancer cell.

15. The method of claim 9, wherein the cancer cell is a renal cancer cell.

16. The method of claim 1, wherein the cancer cell is contacted with a chemotherapeutic drug.

17. The method of claim 16, wherein the chemotherapeutic drug comprises an alkylating agent, mitotic inhibitor, antibiotic, nitrosurea, antimetabolite, corticosteroid hormone, or other antineoplastic agent.

18. The method of claim 17, wherein the chemotherapeutic drug comprises an alkylating agent.

19. The method of claim 17, wherein the chemotherapeutic drug comprises a mitotic inhibitor.

20. The method of claim 17, wherein the chemotherapeutic drug comprises an antibiotic.

21. The method of claim 17, wherein the chemotherapeutic drug comprises a nitrosourea.

22. The method of claim 17, wherein the chemotherapeutic drug comprises an antimetabolite.

23. The method of claim 17, wherein the chemotherapeutic drug comprises a corticosteroid hormone.

24. The method of claim 17, wherein the chemotherapeutic drug comprises an antineoplastic agent.

25. The method of claim 1, wherein the thiazolidinedione compound is contacted with a cancer cell by administering the thiazolidinedione regionally, endoscopically, intravenously, intralesionally, percutaneously, subcutaneously, intraperitoneally, intratracheally, intramuscularly, or by perfusion.

26. The method of claim 17, wherein the thiazolidinedione and the chemotherapeutic drug are suitably dispersed in a pharmacologically acceptable formulation.

27. The method of claim 1, wherein the thiazolidinedione compound is contacted with the cancer cell at the same time as contact with the chemotherapeutic agent.

28. The method of claim 1, wherein the cancer cell is a tumor cell in a tumor.

29. The method of claim 28, further comprising resecting the tumor.

30. The method of claim 28, wherein the cancer cell is irradiated with X-ray irradiation, UV-irradiation, -irradiation, or microwaves.

31. The method of claim 30, wherein the thiazolidinedione compound is contacted with the cancer cell at the same time as irradiation.

32. The method of claim 25, further comprising contacting the cancer cell with a therapeutic polynucleotide selected from the group consisting of a Dp gene, p21, p16, p27, E2F, Rb, APC, DC, NF-1, NF-2, WT-1, MEN-I, MEN-II, BRCA1, VHL, FCC, MCC, *ras*, *myc*, *neu*, *raf*, *erb*, *src*, *fms*, *jun*, *trk*, *ret*, *gsp*, *hst*, *bcl*, *abl*, Bax, Bcl-X_S and E1A; wherein the therapeutic polynucleotide is expressed in the cancer cell.

33. A method for treating cancer in a patient comprising administering to the patient troglitazone and a chemotherapeutic drug in an amount effective to produce a therapeutic benefit.

34. A method for inhibiting the cell cycle progression of a mammalian cancer cell comprising contacting the cell with an amount of troglitazone and a chemotherapeutic drug effective to inhibit the cell cycle progression of the cell.

35. A method of treating cancer in a patient comprising administering to the patient a therapeutically effective amount of troglitazone and a chemotherapeutic drug.

36. A method for treating microscopic residual cancer comprising the steps of:
(i) identifying a patient having a resectable tumor;
(ii) resecting said tumor; and
(iii) contacting the tumor bed with a therapeutically effective amount of troglitazone and a chemotherapeutic drug.

37. A method for treating a subject having a tumor comprising the steps of:
(i) surgically revealing said tumor; and
(ii) contacting said tumor with a therapeutically effective amount of troglitazone and a chemotherapeutic drug.

38. A method for treating a subject having a tumor comprising the step of perfusing said tumor, over an extended period of time, with a therapeutically effective amount of troglitazone and a chemotherapeutic drug.

39. The method of claim 27, wherein the thiazolidinedione and the chemotherapeutic agent are combined in a therapeutic formulation.

40. A method for inhibiting the growth of a cancer cell comprising:
i) contacting the cancer cell with a composition comprising troglitazone; and
ii) contacting the cancer cell with a chemotherapeutic agent or irradiating the cancer cell, in amounts effective to inhibit growth of the cancer cell.

41. The method of claim 40, wherein the cancer cell is contacted with a chemotherapeutic agent.

42. The method of claim 41, wherein the composition comprises troglitazone and a chemotherapeutic agent.

43. The method of claim 40, wherein the cancer cell is a bone cancer cell.

44. The method of claim 43, wherein the bone cancer cell is an osteosarcoma cell.

45. The method of claim 40, wherein the cancer cell is an ovarian cancer cell.

46. The method of claim 40, wherein the cancer cell is a renal cancer cell.

EXHIBIT C

COPY OF PENDING CLAIMS IF AMENDMENT IS NOT ENTERED

1. A method for inhibiting the growth of a cancer cell comprising:
   (i) contacting the cancer cell with a thiazolidinedione compound; and
   (ii) contacting the cancer cell with a chemotherapeutic drug or irradiating the cancer cell with X-ray irradiation, UV-irradiation, γ-irradiation, or microwaves, in amounts effective to inhibit the growth of the cancer cell.

2. The method of claim 1, wherein the thiazolidinedione compound is a troglitazone.

3. The method of claim 1, wherein the thiazolidinedione compound is a pioglitazone.

4. The method of claim 1, wherein the thiazolidinedione compound is a rosiglitazone.

5. The method of claim 1, wherein the cancer cell is a mammalian cancer cell.

6. The method of claim 5, wherein the cancer cell is a human cancer cell.

7. The method of claim 1, wherein the contacting occurs *in vitro*.

8. The method of claim 1, wherein the contacting occurs *in vivo*.

9. The method of claim 1, wherein the cancer cell is selected from a group consisting of a bladder, blood, bone, bone marrow, brain, breast, colon, esophagus, gastrointestine, head, kidney, liver, lung, nasopharynx, neck, ovary, prostate, skin, stomach, and uterus cell.

10. The method of claim 9, wherein the cancer cell expresses PPAR-γ.

11. The method of claim 9, wherein the cancer cell is a bone cancer cell.

12. The method of claim 11, wherein the bone cancer cell is an osteosarcoma cell.

13. The method of claim 11, wherein the cancer cell is a precursor to osteosarcoma.

14. The method of claim 9, wherein the cancer cell is an ovarian cancer cell.

15. The method of claim 9, wherein the cancer cell is a renal cancer cell.

16. The method of claim 1, wherein the cancer cell is contacted with a chemotherapeutic drug.

17. The method of claim 16, wherein the chemotherapeutic drug comprises an alkylating agent, mitotic inhibitor, antibiotic, nitrosurea, antimetabolite, corticosteroid hormone, or other antineoplastic agent.

18. The method of claim 17, wherein the chemotherapeutic drug comprises an alklyating agent.

19. The method of claim 17, wherein the chemotherapeutic drug comprises a mitotic inhibitor.

20. The method of claim 17, wherein the chemotherapeutic drug comprises an antibiotic.

21. The method of claim 17, wherein the chemotherapeutic drug comprises a nitrosourea.

22. The method of claim 17, wherein the chemotherapeutic drug comprises an antimetabolite.

23. The method of claim 17, wherein the chemotherapeutic drug comprises a corticosteroid hormone.

24. The method of claim 17, wherein the chemotherapeutic drug comprises an antineoplastic agent.

25. The method of claim 1, wherein the thiazolidinedione compound is contacted with a cancer cell by administering the thiazolidinedione regionally, endoscopically, intravenously, intralesionally, percutaneously, subcutaneously, intraperitoneally, intratracheally, intramuscularly, or by perfusion.

26. The method of claim 17, wherein the thiazolidinedione and the chemotherapeutic drug are suitably dispersed in a pharmacologically acceptable formulation.

27. The method of claim 1, wherein the thiazolidinedione compound is contacted with the cancer cell at the same time as contact with the chemotherapeutic agent.

28. The method of claim 1, wherein the cancer cell is a tumor cell in a tumor.

29. The method of claim 28, further comprising resecting the tumor.

30. The method of claim 28, wherein the cancer cell is irradiated with X-ray irradiation, UV-irradiation, -irradiation, or microwaves.

31. The method of claim 30, wherein the thiazolidinedione compound is contacted with the cancer cell at the same time as irradiation.

32. The method of claim 25, further comprising contacting the cancer cell with a therapeutic polynucleotide selected from the group consisting of a Dp gene, p21, p16, p27, E2F, Rb, APC, DC, NF-1, NF-2, WT-1, MEN-I, MEN-II, BRCA1, VHL, FCC, MCC, *ras*, *myc*, *neu*, *raf*, *erb*, *src*, *fms*, *jun*, *trk*, *ret*, *gsp*, *hst*, *bcl*, *abl*, Bax, Bcl-X_S and E1A; wherein the therapeutic polynucleotide is expressed in the cancer cell.

33. A method for treating cancer in a patient comprising administering to the patient troglitazone and a chemotherapeutic drug in an amount effective to produce a therapeutic benefit.

34. A method for inhibiting the cell cycle progression of a mammalian cancer cell comprising contacting the cell with an amount of troglitazone effective to inhibit the cell cycle progression of the cell.

35. A method of treating cancer in a patient comprising administering to the patient a therapeutically effective amount of troglitazone and a chemotherapeutic drug.

36. A method for treating microscopic residual cancer comprising the steps of:
(i) identifying a patient having a resectable tumor;
(ii) resecting said tumor; and
(iii) contacting the tumor bed with a therapeutically effective amount of troglitazone and a chemotherapeutic drug.

37. A method for treating a subject having a tumor comprising the steps of:
(i) surgically revealing said tumor; and
(ii) contacting said tumor with a therapeutically effective amount of troglitazone and a chemotherapeutic drug.

38. A method for treating a subject having a tumor comprising the step of perfusing said tumor, over an extended period of time, with a therapeutically effective amount of troglitazone and a chemotherapeutic drug.

39. The method of claim 27, wherein the thiazolidinedione and the chemotherapeutic agent are combined in a therapeutic formulation.

40. A method for inhibiting the growth of a cancer cell comprising:
i) contacting the cancer cell with a composition comprising troglitazone; and
ii) contacting the cancer cell with a chemotherapeutic agent or irradiating the cancer cell, in amounts effective to inhibit growth of the cancer cell.

41. The method of claim 40, wherein the cancer cell is contacted with a chemotherapeutic agent.

42. The method of claim 41, wherein the composition comprises troglitazone and a chemotherapeutic agent.

43. The method of claim 40, wherein the cancer cell is a bone cancer cell.

44. The method of claim 43, wherein the bone cancer cell is an osteosarcoma cell.

45. The method of claim 40, wherein the cancer cell is an ovarian cancer cell.

46. The method of claim 40, wherein the cancer cell is a renal cancer cell.

US005814647A

United States Patent [19]

Urban et al.

[11] Patent Number: 5,814,647 A4

[45] Date of Patent: Sep. 29, 1998

[54] **USE OF TROGLITAZONE AND RELATED COMPOUNDS FOR THE TREATMENT OF THE CLIMACTERIC SYMPTOMS**

[75] Inventors: **Randall J. Urban**, Friendswood; **Allan Green**, Galveston, both of Tex.

[73] Assignee: **Board of Regents, The University of Texas System**, Austin, Tex.

[21] Appl. No.: **811,419**

[22] Filed: **Mar. 4, 1997**

[51] **Int. Cl.**[6] **A61K 31/44**; A61K 31/425; A61K 31/41

[52] **U.S. Cl.** **514/369**; 514/252; 514/256; 514/342; 514/360; 514/375; 514/376

[58] **Field of Search** 514/252, 256, 514/342, 360, 369, 375, 376

[56] **References Cited**

U.S. PATENT DOCUMENTS

5,478,852 12/1995 Olefsky et al. 514/369

OTHER PUBLICATIONS

Barentsen R. Eur J Obs Gyn & Repr Biol 64 Suppl:s7–11. 1996.

Berge et al., "Pharmaceutical Salts." *J. Pharm. Science*, 66:1–19, 1977.

Connelly EF, Budd GT. Seminars in Oncology 23:16–21. 1996.

Dunaif, Scott, Finegood, Quintana, Whitcomb. "The insulin–sensitizing agent Troglitazone improves metabolic and reproductive abnormalities in the polycystic ovary syndrome." *J. Clin. Endocrinol. Metab.*, 81:3299–3306. 1996.

Forman, Tontonoz, Chen, Brun, Spiegelman, Evans. "15–Deoxy–delta^{12,14}–Prostaglandin J_2 is a ligand for the adipocyte determination factor PPAR gamma." *Cell*, 83:803–812. 1995.

Iwamoto et al., "Effects of Troglitazone: A New Hypoglycemic Agent in Patients with NIDDM Poorly Controlled by Diet Therapy." Diabetes Care, 19(2):151–156. 1996.

Kellerer et al., "Troglitazone Prevents Glucose–Induced Insulin Resistance of Insulin Receptor in Rat–1 Fibroblasts." Diabetes, 43:447–453. 1994.

Kliewer, Lenhard, Wilson, Patel, Morris, Lehman. "A prostaglandin J_2 metabolite binds peroxisome proliferator–activated receptor gamma and promotes adipocyte differentiation." *Cell*, 83:813–819. 1995.

Lehmann, Moore, Smith–Oliver, Wilkison, Willson, Kliewer. "An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator–activated receptor gamma (PPARgamma)." *J. Biol. Chem.*, 270:12953–12956. 1995.

Marcus, Capone, Rachubinski. "Identification of COUP–T-FII as a peroxisome proliferator response element binding factor using genetic selection in yeast: COUP–TFII activates transcription in yeast but antagonizes PPAR signaling in mammalian cell." *Mol. Cell. Endocrinol.*, 120:31–39. 1996.

Miller. "Molecular biology of steroid hormone synthesis." *Endocr. Rev.*, 9:295–318. 1988.

Motojima. "Peroxisome proliferator–activated receptor (PPAR): Structure, mechanisms of activation and diverse functions." *Cell Structure and Function*. 18:267–277. 1993.

Nolan, Ludvik, Beerdsen, Joyce, Olefsky. "Improvement in glucose tolerance and insulin resistance in obese subjects treated with Troglitazone." *N. Engl. J. Med.*, 331:1188–1193. 1994.

Odell PF. Journal of Otolaryngology 25:7–13. 1996.

Pierce WC. Figlin RA. Curr Opin Oncology 5:343–352. 1992.

Rainey, Sawetawan, Shay, Michael, Mathis, Kutteh, Byrd, Carr. "Transformation of human granulosa cells with the E6 and E7 regions of human papillomavirus." *J. Clin. Endocrinol. Metab.*, 78:705–710. 1994.

Shmookler et al., "Giant Cell Fibroblastoma : A Juvenile Form of Dermatofibrosarcoma Protuberans." Cancer, 64:2154–2161. 1989.

Shaaban MM. "The perimenopause and contratception" Maturitas 23:181–192. 1996.

Sutter et al., "Metabolic Effects of New Oral Hypoglycemic Agent CS–045 in NIDDM Subjects." Diabetes Care, 15(2):193–203. 1992.

Tontonoz et al., "Terminal Differentiation of Human Liposarcoma Cells Induced by Ligands for Peroxisome Proliferator–Activated Receptor γ and the Retinoid X Receptor." Proc. Natl. Acad. Sci. USA, 94:237–241.1997.

Urban, Bodenburg, Nagamani, Peirce. "Dexamethasone potentiates IGF–I actions in porcine granulosa cells." *Am. J. Physiol.*, 267:E115–E123. 1994.

Urban, Garmey, Shupnik, Veldhuis. "Insulin–like growth factor type I increases concentrations of messenger RNA encoding cytochrome P450 cholesterol side chain cleavage enzyme in primary cultures of porcine granulosa cells." *Endocrinology*, 127:2481–2488. 1990.

Veldhuis and Furlanette, "Trophic actions of human somatomedin C/insulin–like growth facrtor I on ovarian cells: in vitro studies with swine granulosa cells." *Endocrinology*, 116:1235–1242. 1985.

Vidal–Puig, Jimenez, Liñan, Lowell, Hamann, Hu, Spiegelman, Flier, Moller. "Regulation of PPAR gamma gene expression by nutrition and obesity in rodents." *J. Clin. Invest.*, 97:2553–2561. 1996.

Primary Examiner—Jerome D. Goldberg
Attorney, Agent, or Firm—Arnold White & Durkee

[57] **ABSTRACT**

The present invention is directed toward the use of the drug Troglitazone and related thiazolidinedione compounds in the treatment of the climacteric and cancer. This use is based on the novel discovery that Troglitazone inhibits steroidogenesis in granulosa cell cultures. This activity is believed to result from the ability of thiazolidinedione derivatives to act as a ligand for the orphan steroid receptor peroxisome proliferator-activated receptor gamma (PPARγ). Troglitazone and related compounds can therefore be used to prevent excessive uterine bleeding during. Further, enhanced translocation of this orphan nuclear receptor into the nucleus of cells will block transcription in rapidly proliferating cancer cells that express PPARγ, resulting in loss of cell viability.

24 Claims, 14 Drawing Sheets

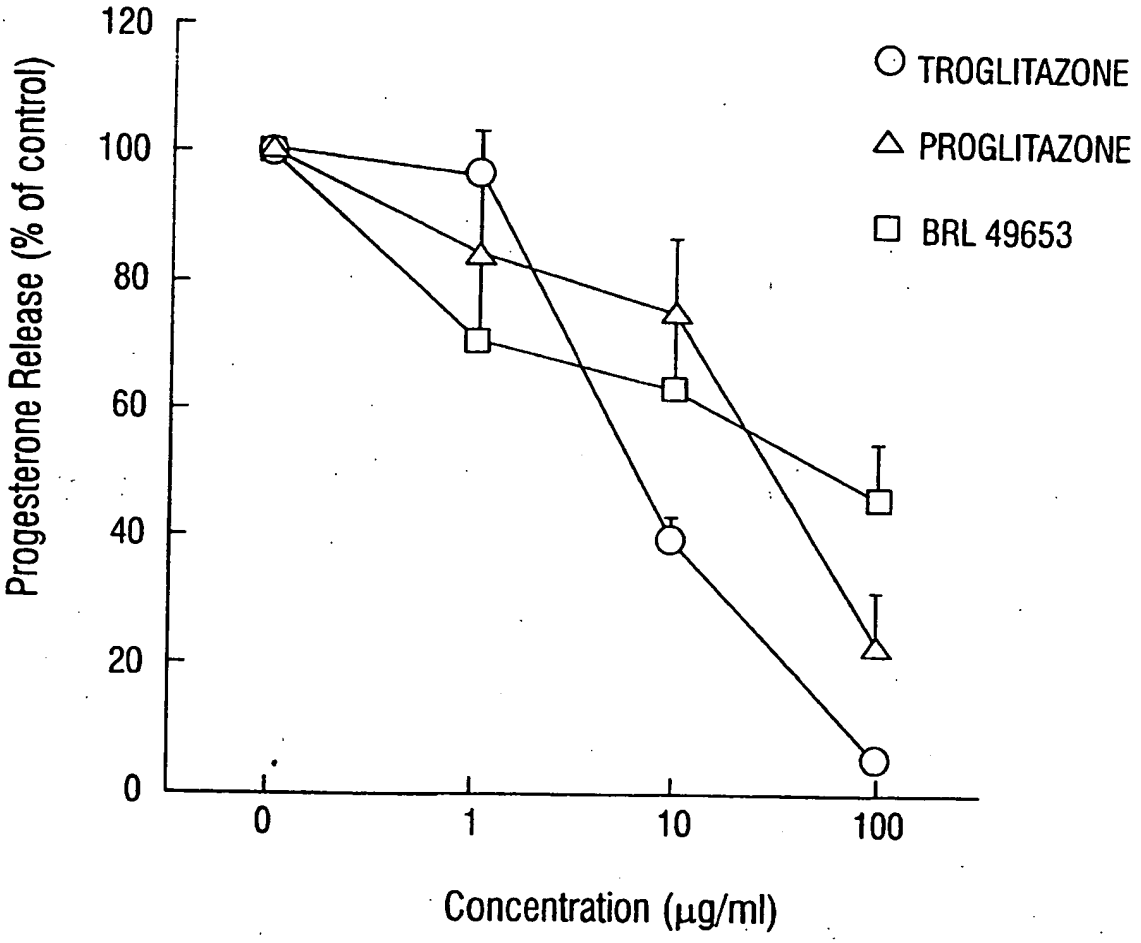

120
100
80
60
40
20
0
Progesterone Release (% of control)
0
1
10
100
Concentration (μg/ml)
TROGLITAZONE
PROGLITAZONE
BRL 49653

FIG. 1

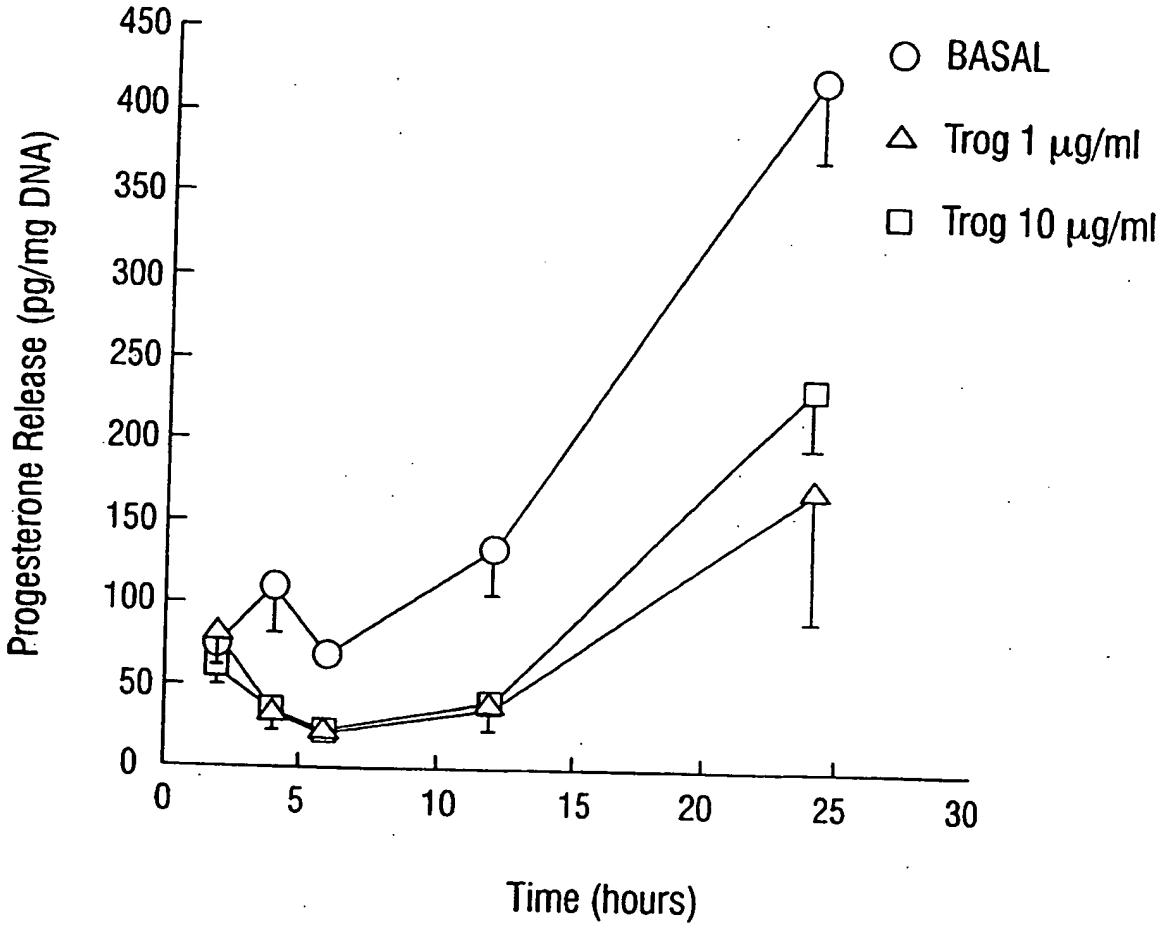

Progesterone Release (pg/mg DNA)
450
400
350
300
250
200
150
100
50
0
0
5
10
15
20
25
30
Time (hours)
BASAL
Trog 1 µg/ml
Trog 10 µg/ml

FIG. 2

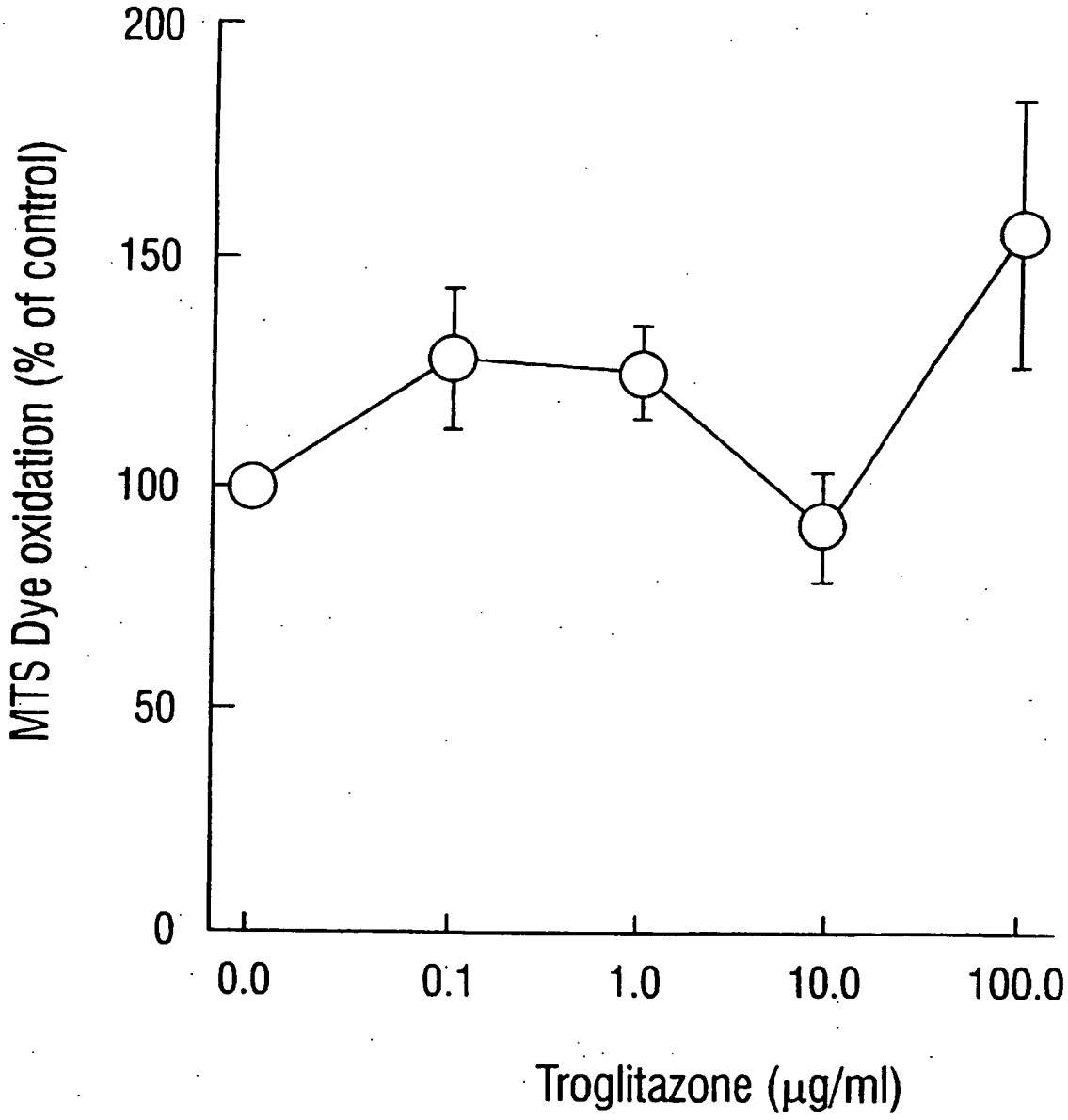

200
150
100
50
0
MTS Dye oxidation (% of control)
0.0
0.1
1.0
10.0
100.0
Troglitazone (μg/ml)

FIG. 3

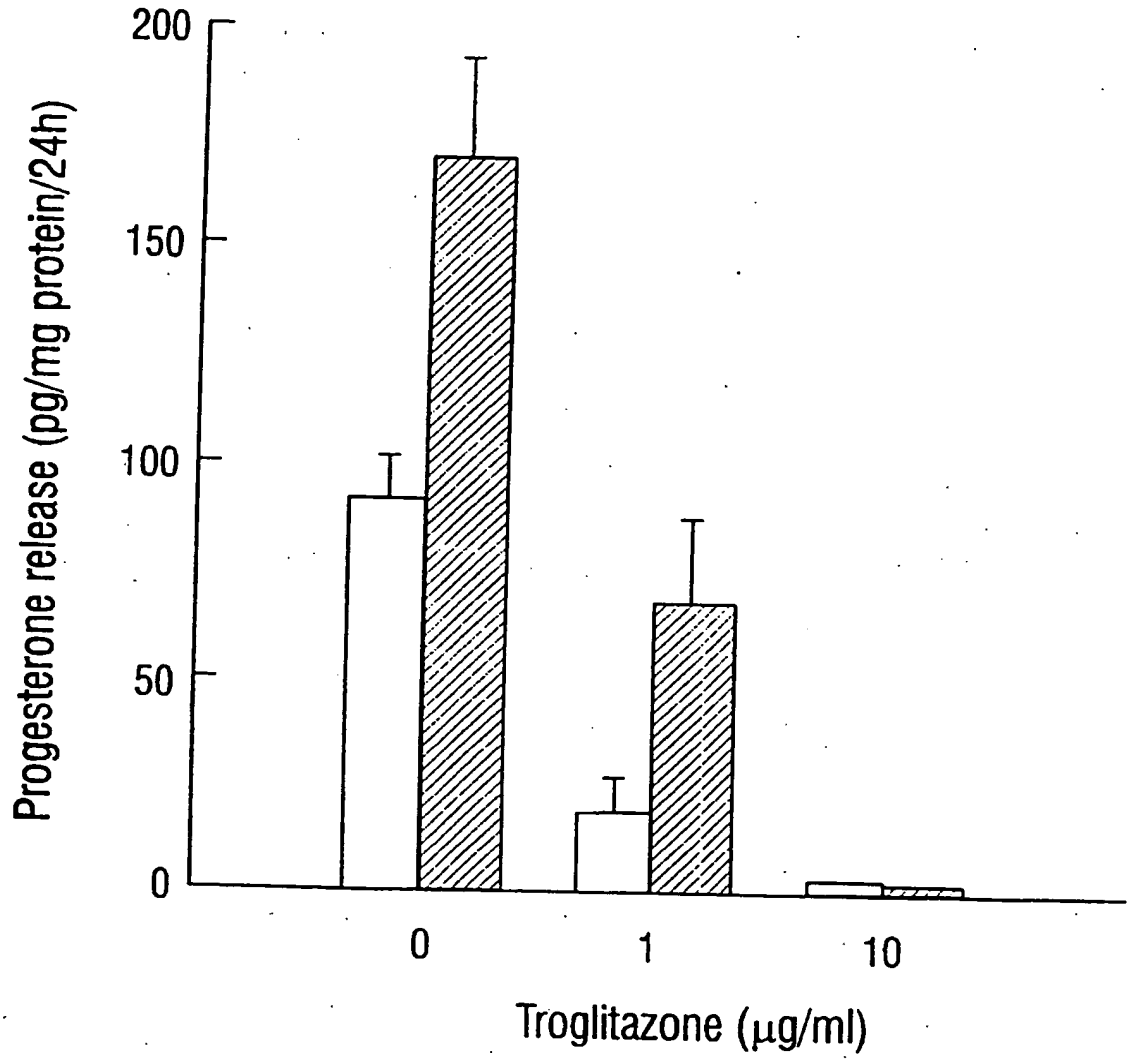

200
150
100
50
0
Progesterone release (pg/mg protein/24h)
0
1
10
Troglitazone (μg/ml)

FIG. 4

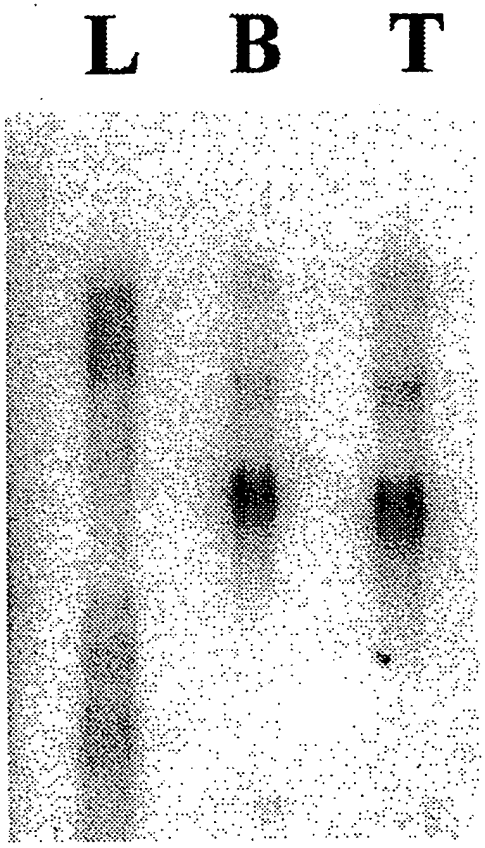


FIG. 5

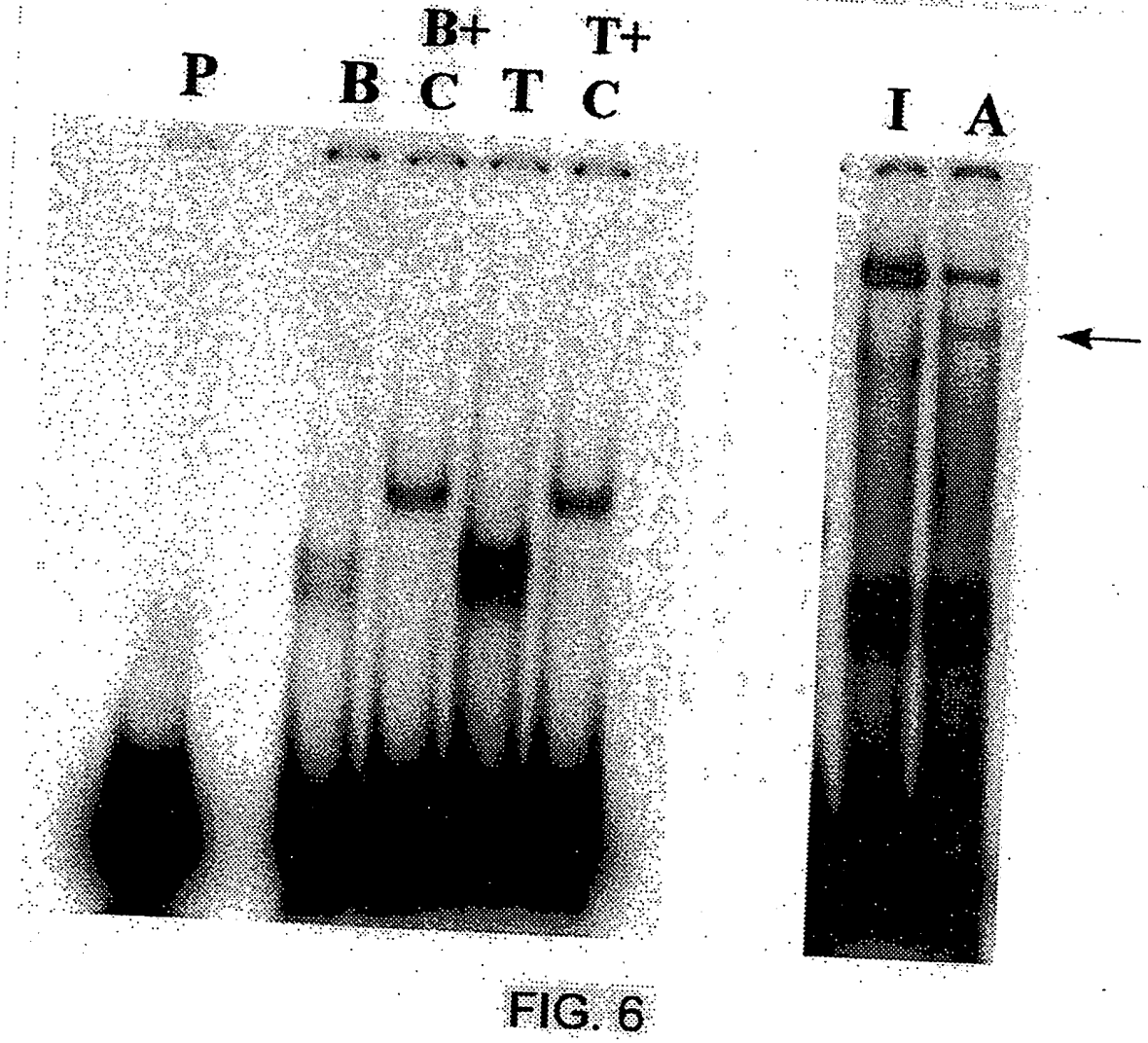


FIG. 6

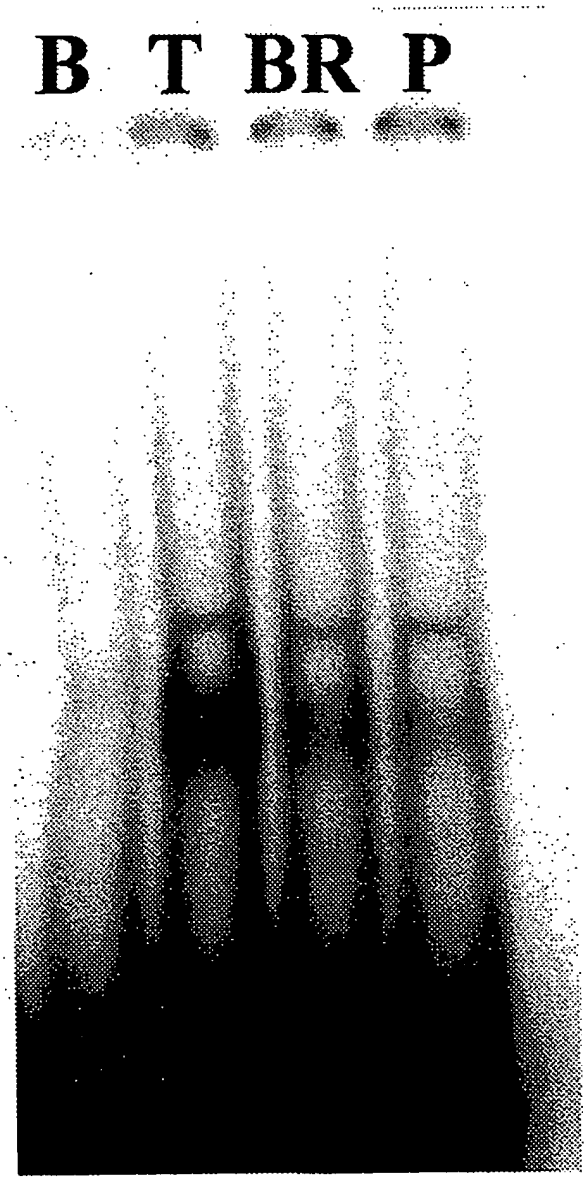


FIG. 7A

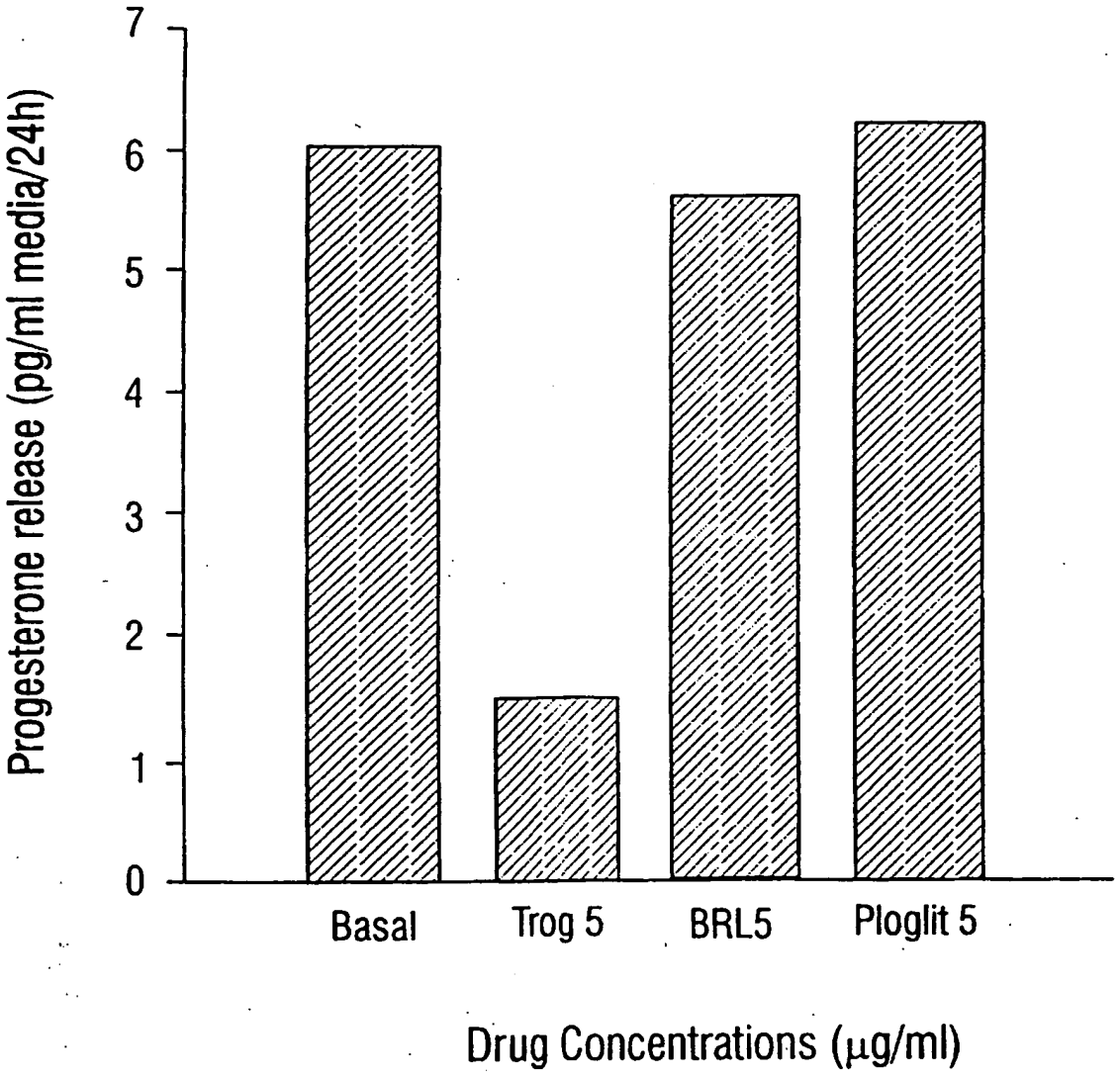

Progesterone release (pg/ml media/24h)
0
1
2
3
4
5
6
7
Basal
Trog 5
BRL5
Ploglit 5
Drug Concentrations (μg/ml)

FIG. 7B

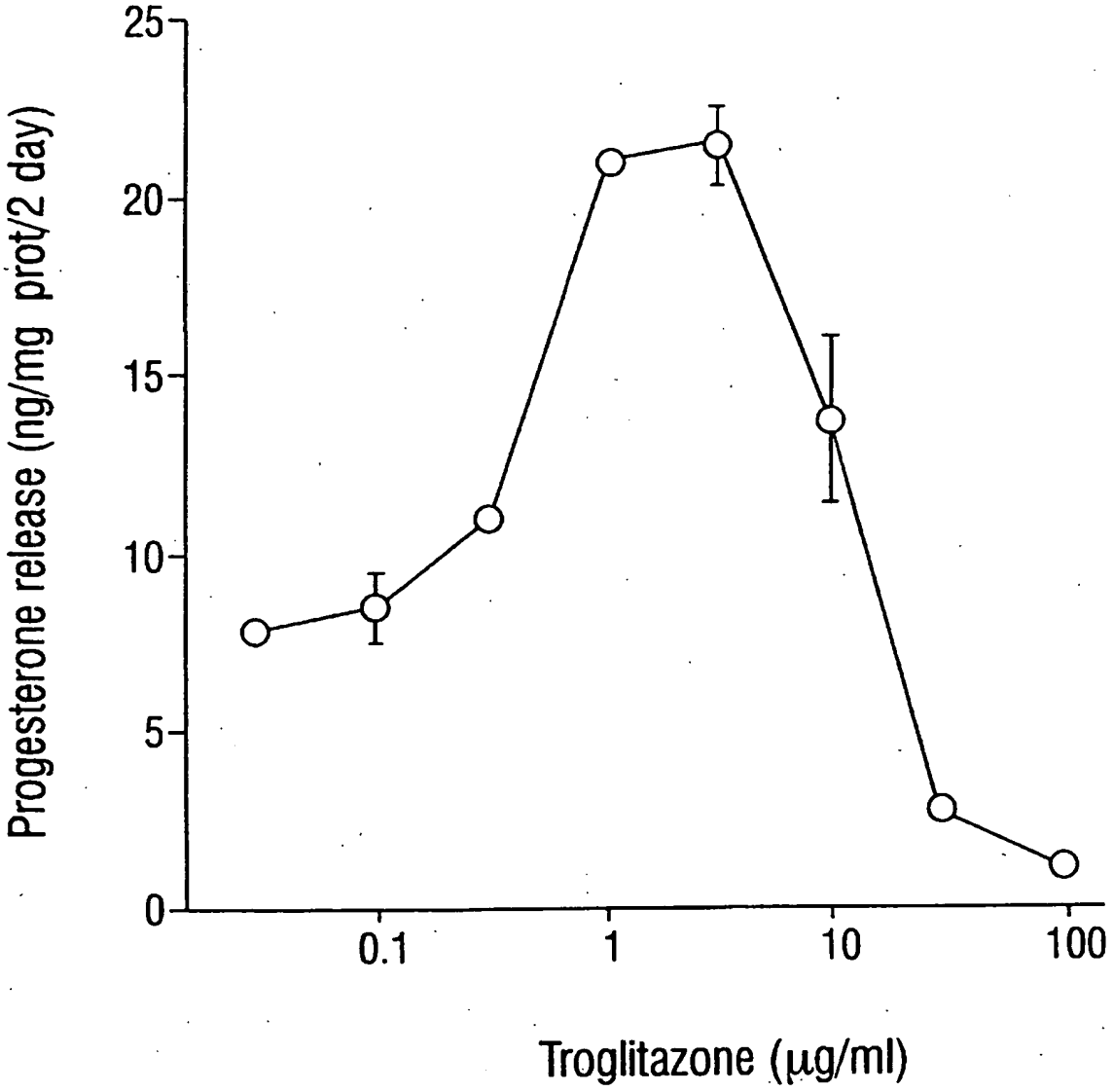

Progesterone release (ng/mg prot/2 day)
25
20
15
10
5
0
0.1
1
10
100
Troglitazone (μg/ml)

FIG. 8

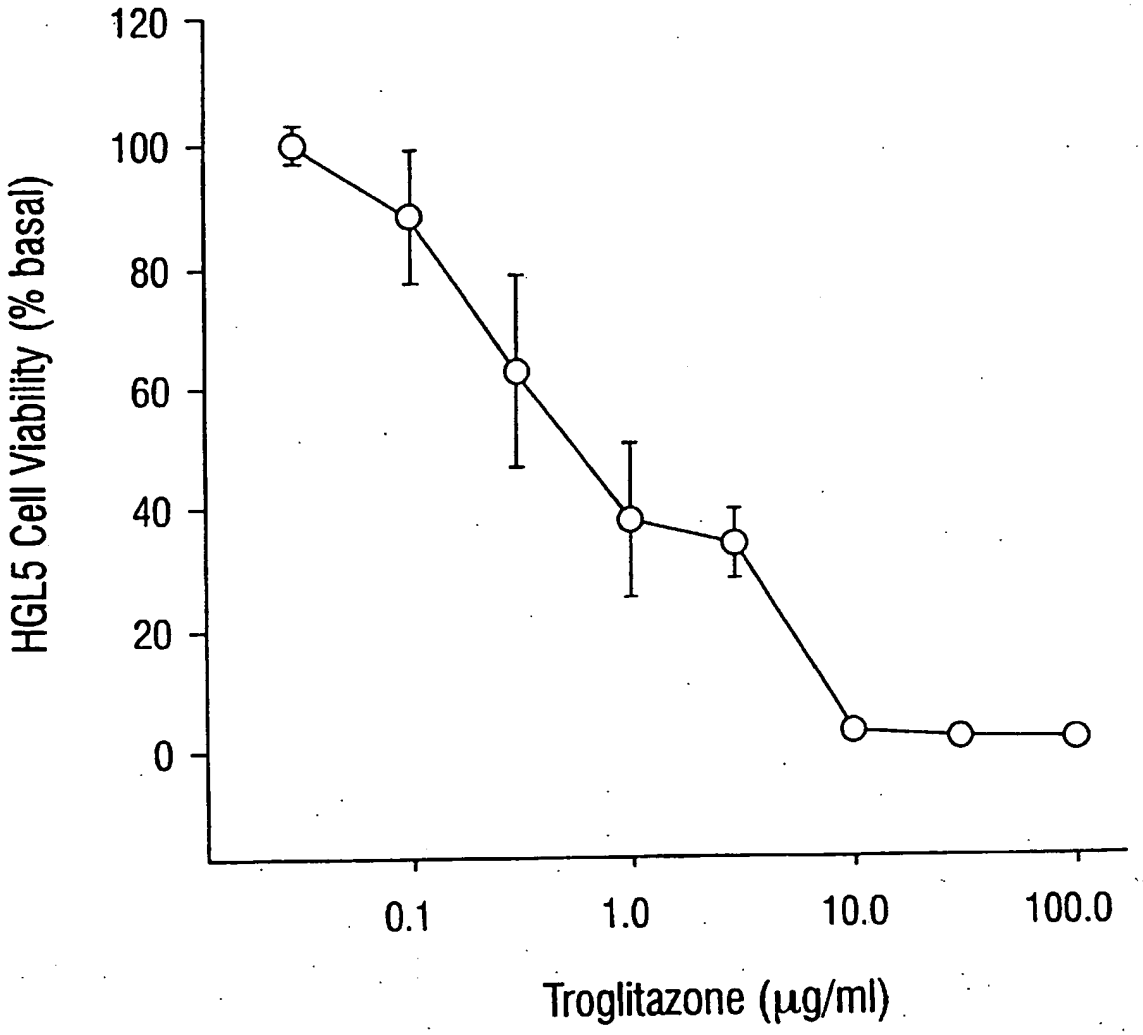

120
100
80
60
40
20
0
HGL5 Cell Viability (% basal)
0.1
1.0
10.0
100.0
Troglitazone (μg/ml)

FIG. 9

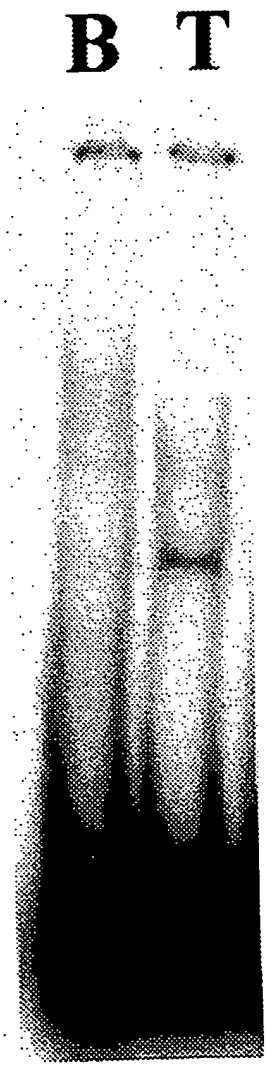


FIG. 10

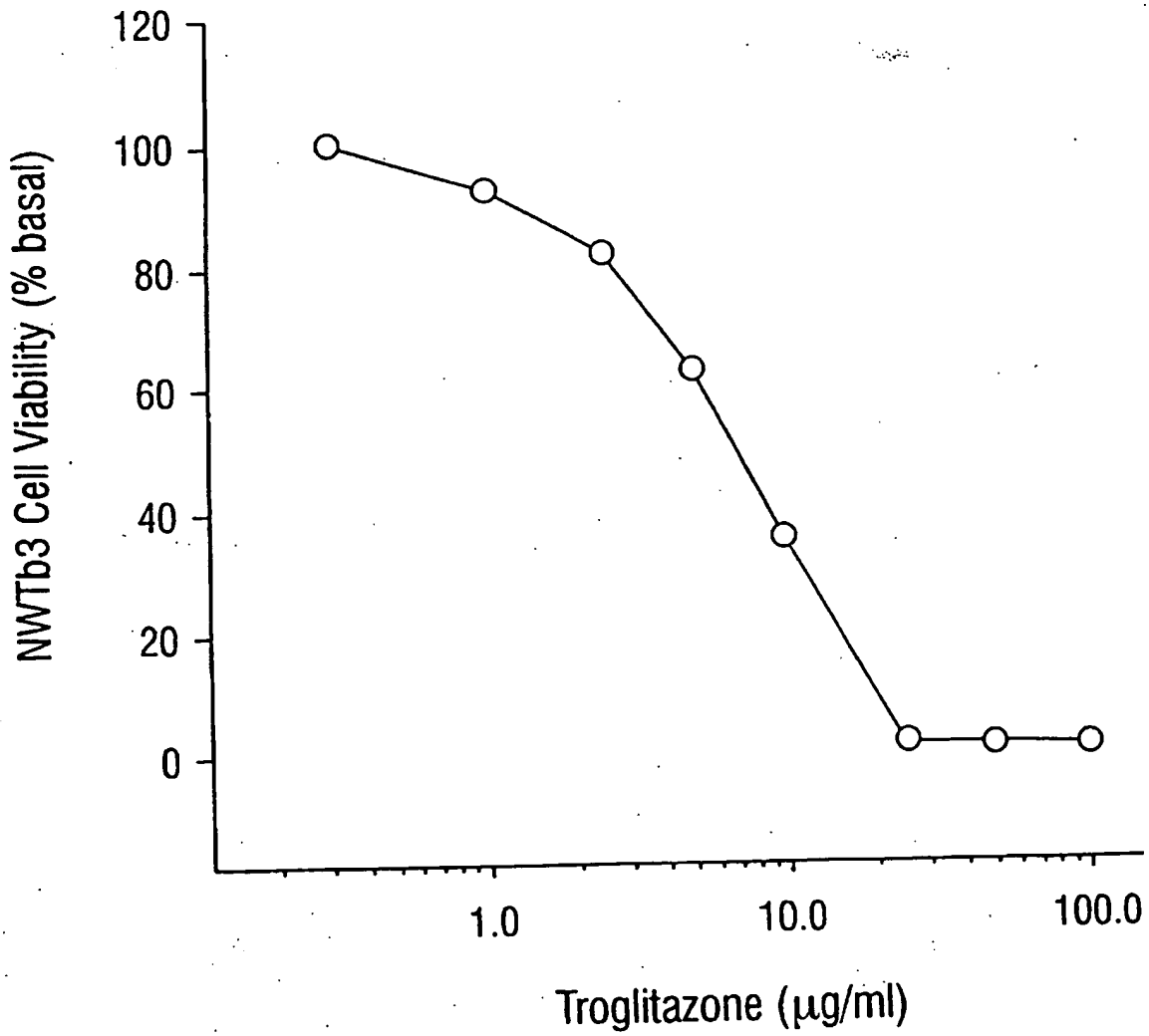

120
100
80
60
40
20
0
NWTb3 Cell Viability (% basal)
1.0
10.0
100.0
Troglitazone (µg/ml)

FIG. 11

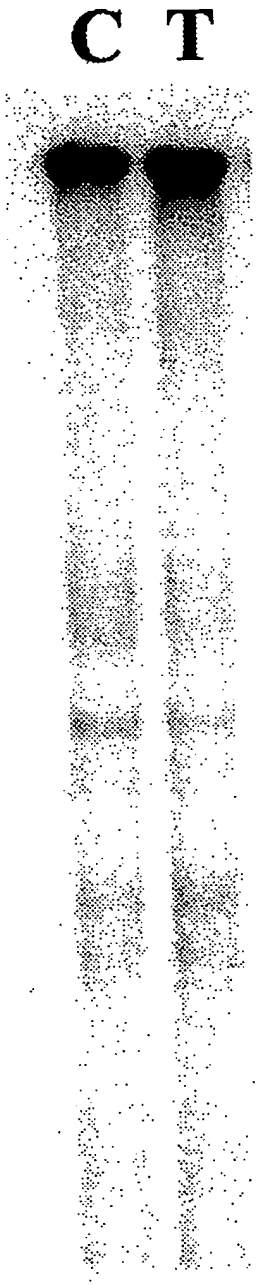


FIG. 12

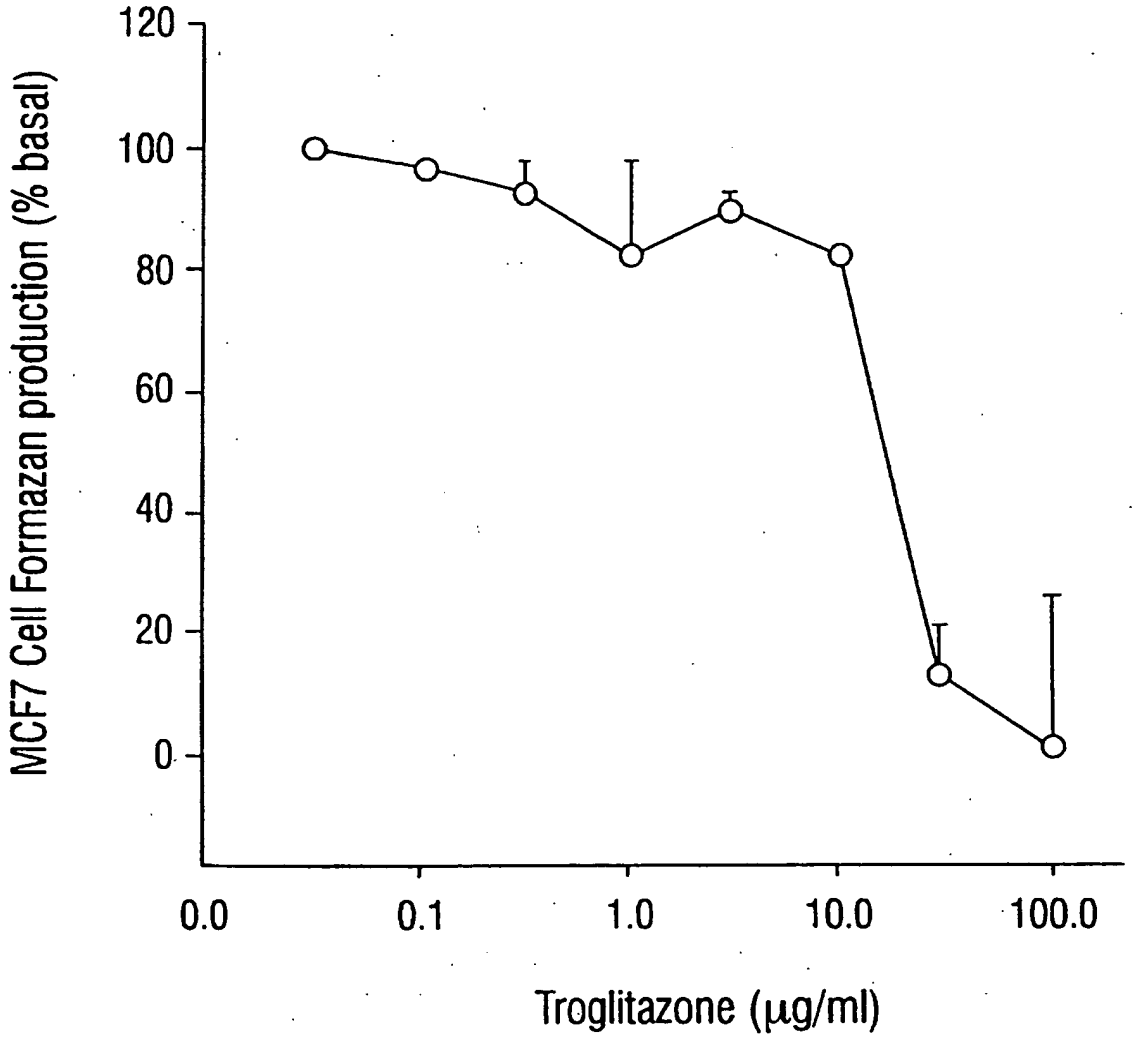

120
100
80
60
40
20
0
MCF7 Cell Formazan production (% basal)
0.0
0.1
1.0
10.0
100.0
Troglitazone (μg/ml)

FIG. 13

USE OF TROGLITAZONE AND RELATED COMPOUNDS FOR THE TREATMENT OF THE CLIMACTERIC SYMPTOMS

The Federal government has rights in the present invention insofar as it was supported by NIH grant No. 1-R01-HD28393.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates generally to a novel use of Troglitazone and related thiazolidinedione derivatives. More specifically, it relates to the use of Troglitazone and related thiazolidinedione derivatives in the treatment of the climacteric. It also relates to the use of Troglitazone and the like for the treatment of cancer.

2. Description of Related Art

Troglitazone is a member of a class of antidiabetic drugs termed thiazolidinediones. Although the mechanism is unknown, this class of drugs lowers insulin resistance and improves glucose tolerance (Nolan el al., 1994). For these reasons Troglitazone has found use in the treatment of noninsulin-dependent diabetes mellitus (NIDDM) (U.S. Pat. No. 5,478,852 incorporated by reference herein). NIDDM, otherwise referred to as Type II diabetes, is the form of diabetes mellitus which occurs predominately in adults in whom adequate production of insulin is available for use, yet a defect exists in insulin-mediated utilization and metabolism of glucose in peripheral tissues. The population with impaired glucose tolerance progresses to NIDDM at a rate of 5% to 10% of cases per year. Failure to treat NIDDM can result in mortality due to cardiovascular disease and other diabetic complications including retinopathy, neplhropathy, and peripheral neuropathy. Administration of Troglitazone can provide effective treatment of populations experiencing impaired glucose tolerance and may result in the delay or prevention of the onset of NIDDM.

Moreover, Troglitazone has been further implicated in the treatment of polycystic ovary syndrome (PCO). This is a syndrome in women that is characterized by chronic anovulation and hyperandrogenism. Women with this syndrome often have insulin resistance and an increased risk for the development of noninsulin-dependent diabetes mellitus. In women with PCO given Troglitazone (400 mg every day), insulin resistance was reduced and 2 of the 25 women studied had ovulatory menses (Dunaif et al., 1996).

While advances continue to be made in chemotherapy treatment of cancer, effective agents are still lacking for the treatment many types of cancer. One such type is mesenchymal tumors. The mesenchyma consists of the meshwork of embryogenic connective tissue in the mesoderm from which are formed the connective tissues of the body as well as blood vessels and lymphatic vessels. There are many types of mesenchymal tumors including but not limited to sarcomas (general), rhabdomyosarcomas, fibrosarcomas, retinoblastoma, hemangiopericytoma, congenital mesoblastic nephroma, and mesotheliomas (Pierce and Figlin, 1992; Odell, 1996; Connelly and Budd, 1996). These types of tumors are aggressive and fast growing, thus development of effective chemotherapeutic agents for their treatment is of particular need.

One mechanism through which thiazolidinediones are believed to have biological effect is their ability to serve as a high affinity ligand for the orphan steroid receptor peroxisome proliferator-activated receptor gamma (PPARγ) (Lehmann et al., 1995). PPARγ is a member of the nuclear

receptor superfamily of orphan steroid receptors that serve as transcription factors (Motojima, 1993). This family includes receptors for the steroid, thyroid and retinoid hormones. Activation of PPARγ is implicated in adipocyte differentiation through the activation of adipocyte-specific gene expression (Lehmann et al., 1995). This gene expression is mediated through binding to a PPARγ response element (PPRE) in the promoter region of target genes (Forman et al., 1995). This PPRE is composed of a directly repeating core site separated by one nucleotide (NNN-AGGTCA-N-AGGTCA). To bind to a PPRE, PPARγ must form a heterodimer with the 9-cis retinoic acid receptor (RXR). This sequence is classified as a DR-1 consensus sequence that is universal for orphan receptors (Vidal-Puig, 1996). Because of the universal nature of this consensus sequence, other transcription factors can bind to the PPRE and compete with the binding of PPARγ. One such transcription factor is COUP-TFII that antagonizes PPAR signaling in mammalian cells (Marcus et al., 1996).

PPARγ is in a family of three orphan receptors that are encoded by different genes (Motojima, 1993). The three PPAR genes are PPARα, PPARδ, and PPARγ (Motojima, 1993). Moreover, 2 isoforms of PPARγ also exist, PPARγ1 and γ2 (Vidal-Puig et al., 1996). These 2 proteins differ only in their NH_2-terminal-30 amino acids and are the result of alternative promoter usage and differential mRNA splicing (Vidal-Puig et al., 1996). In addition to thiazolidinediones, another ligand for the PPARγ nuclear receptor is the arachidonic acid metabolite 15-deoxy-delta^{12,14}-prostaglandin J_2 (15d-PGJ_2). This prostaglandin activates PPARγ-dependent adipogenesis, but activated PPARα only at high concentrations (Forman et al., 1995; Kliewer et al., 1995). This is further evidence that the PPAR family subtypes are distinct from one another in their pharmacological response to ligands

The climacteric is defined as the syndrome of endocrine, somatic and psychological changes occurring at the termination of the reproductive period in the female. The menstrual irregularities are episodes of prolonged menstrual bleeding caused by a loss of ovulation. The loss of ovulation is caused by a failure of development of ovarian follicles. Currently the most common method for treatment of the climacteric is hormone replacement, including administration of birth control pills, oral administration of estrogen and progesterone preparations or oral administration of progesterone only preparations (Shaaban, 1996). While relieving symptoms of the climacteric, these treatments have many associated risks and side effects. Risks associated with hormone treatment include endometrial carcinoma, hypertension, hyperlipidemia, cholelithiasis (gallstones), breast cancer, and deep venous thrombosis (Barentsen, 1996).

Compounds useful for practicing the present invention, and methods of making these compounds are known. Some of these compounds are disclosed in WO 91/07107; WO 92/02520; WO 94/01433; WO 89/08651; JP Kokai 69383/92; U.S. Pat. Nos. 4,287,200; 4,340,605; 4,438,141; 4,444,779; 4,461,902; 4,572,912; 4,687,777; 4,703,052; 4,725,610; 4,873,255; 4,897,393; 4,897,405; 4,918,091; 4,948,900; 5,002,953; 5,061,717; 5,120,754; 5,132,317; 5,194,443; 5,223,522; 5,232,925; and 5,260,445. The disclosure of these publications are incorporated herein by reference in particular with respect to the active compounds disclosed therein, and methods of preparation thereof.

SUMMARY OF THE INVENTION

The present invention is the result of the surprising finding that Troglitazone and related thiazolidinedione com-

pounds inhibit steroidogenesis in granulosa cells. A related aspect of the present invention is the discovery that therapeutic levels of Troglitazone can kill rapidly growing cancerous cells expressing the orphan nuclear receptor PPARγ, while not affecting the viability of normal cells. The discovery of these new uses for Troglitazone and related compounds provides important new agents for the treatment of certain types of cancers and for the treatment of the climacteric. The term climacteric is well known in the art as the syndrome of endocrine, somatic and psychological changes occurring at the termination of the reproductive period in the female.

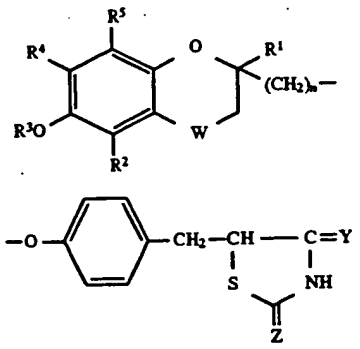
A type of cancer which is particularly likely to be treatable with troglitazone and related thiazolidinedione derivatives are mesenchymal tumors. The mesenchyma consists of the meshwork of embryogenic connective tissue in the mesoderm from which are formed the connective tissues of the body as well as blood vessels and lymphatic vessels. There are many types of mesenchymal tumors including but not limited to sarcomas (general), rhabdomyosarcomas, fibrosarcomas, retinoblastoma, hemangiopericytoma, congenital mesoblastic nephroma, and mesotheliomas.

Studies show that Troglitazone is a ligand for the orphan nuclear receptor PPARγ. Translocation of this transcription factor in the nucleus of cells at sufficient rates inhibits transcription and reduces progesterone production in normal granulosa cells without a loss in cell viability. However, this inhibition of transcription in rapidly dividing cancer cells expressing PPARγ results in the loss of cell viability and inhibition of cell growth. The mechanism of PPARγ inhibition of gene transcription most likely results from the competition of the PPARγ transcription factor with the other orphan nuclear factors binding to DR-1 consensus elements on genes and impairing the promoter activity of those elements. The inhibitory effects of Troglitazone on steroidogenesis make it useful for the reduction of menstrual bleeding in women as they develop reduced ovulation as they approach menopause. Because there is no loss of viability in normal cells, but a reduction in rapidly growing cancer cells, Troglitazone and related compounds may also be used in the treatment of cancer, to impair the growth of cancer cells without killing normal cells

As agents having the aforementioned effects the compounds of the following formulas are useful in treating individuals.

Accordingly, the present invention is the use of compounds of Formula I

Formula I



wherein R^1 and R^2 are the same or different and each represents a hydrogen atom or a C_1–C_5 alkyl group;

R^3 represents a hydrogen atom, a C_1–C_6 aliphatic acyl group, an alicyclic acyl group, an aromatic acyl group, a heterocyclic acyl group, an araliphatic acyl group, a (C_1–C_6 alkoxy)carbonyl group, or an aralkyloxycarbonyl group;

R^4 and R^5 are the same or different and each represents a hydrogen atom, a C_1–C_5 alkyl group or a C_1–C_5 alkoxy group, or R^4 and R^5 together represent a C_1–C_5 alkylenedioxy group;

n is 1, 2 or 3;

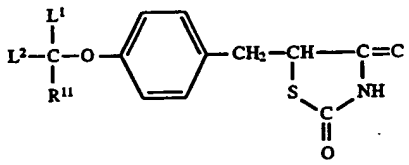
W represents the —CH_2—, >CO, or CH—OR^6 group (in which R^6 represents any one of the atoms or groups defined for R^3 and may be the same as or different from R^3); and

Y and Z are the same or different and each represents an oxygen atom or an imino (=NH) group;

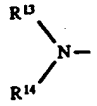
and pharmaceutically acceptable salts thereof.

The present invention is also the use of compounds of the Formula II

Formula II



wherein R^{11} is substituted or unsubstituted alkyl, alkoxy, cycloalkyl, phenylalkyl, phenyl, aromatic acyl group, a 5- or 6-membered heterocyclic group including 1 or 2 heteroatoms selected from the group consisting of nitrogen, oxygen, and sulfur, or a group of the formula



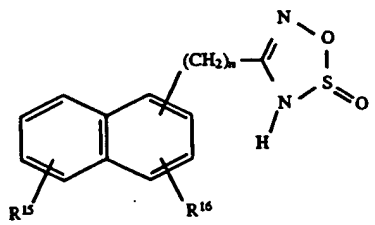
Possible Formula Used for R^{11}

wherein R^{13} and R^{14} are the same or different and each is lower alkyl or R^{13} and R^{14} are combined to each other either directly or as interrupted by a heteroatom selected from the group consisting of nitrogen, oxygen, and sulfur to form a 5- or 6- membered ring;

wherein R^{12} means a bond or lower alkylene group; and wherein L^1 and L^2 are the same or different and each is hydrogen or lower alkyl or L^1 and L^2 are combined to form an alkylene group; or a pharmaceutically acceptable salt thereof.

The present invention is also the use of compounds of the Formula III

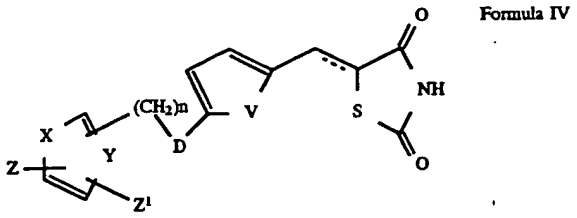
Formula III



wherein R^{15} and R^{16} are independently hydrogen, lower alkyl containing 1 to 6 carbon atoms, alkoxy containing 1 to 6 carbon atoms, halogen, ethynyl, nitrile, methylthio, trifluoromethyl, vinyl, nitro, or halogen substituted benzyloxy; n is 0 to 4 and the pharmaceutically acceptable salts thereof.

The present invention is also directed to the use of compounds of the Formula IV

Formula IV



wherein the dotted line represents a bond or no bond;

V is —H=CH—, —N=CH—, —CH=N— or S;

D is CH_2, CHOH, CO, $C{=}NOR_{17}$ or CH=CH;

X is S, O, NR^{18}, —CH=N or —N=CH;

Y is CH or N;

Z is hydrogen, $(C_1–C_7)$alkyl, $(C_1–C_7)$cycloalkyl, phenyl, naphthyl, pyridyl, furyl, thienyl, or phenyl mono- or disubstituted with the same or different groups which are $(C_1–C_3)$alkyl, trifluoromethyl, $(C_1–C_3)$alkoxy, fluoro, chloro, or bromo;

Z^1 is hydrogen or $(C_1–C_3)$alkyl;

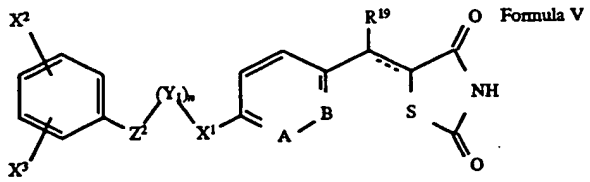
R^{17} and R^1 are each independently hydrogen or methyl; and

n is 1, 2, or 3;

the pharmaceutically acceptable cationic salts thereof; and the pharmaceutically acceptable acid addition salts thereof when the compound contains a basic nitrogen.

The present invention is also directed to the use of compounds of the Formula V

Formula V



wherein the dotted line represents a bond or no bond;

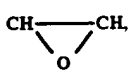
A and B are each independently CH or N, with the proviso that when A or B is N, the other is CH;

X^1 is S, SO, SO_2, CH_2, CHOH, or CO;

n is 0 or 1;

Y^1 is CHR^{20} or R^{21}, with the proviso that when n is 1 and Y^1 is NR^{21}, X^1 is SO_2 or CO;

Z^2 is CHR^{22}, CH_2CH_2, CH=CH,



Possible Formula for Z^2

OCH_2, SCH_2, $SOCH_2$ or SO_2CH_2;

R^{19}, R^{20}, R^{21}, and R^{22} are each independently hydrogen or methyl; and

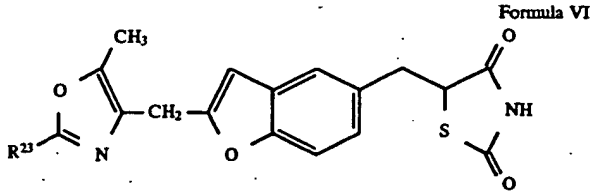
X^2 and X^3 are each independently hydrogen, methyl, trifluoromethyl, phenyl, benzyl, hydroxy, metlhoxy, phenoxy, benzyloxy, bromo, chloro, or fluoro;

a pharmaceutically acceptable cationic salt thereof; or

a pharmaceutically acceptable acid addition salt thereof when A or B is N.

The present invention also relates to the use of compounds of the Formula VI

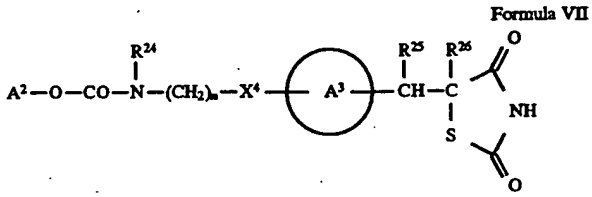
Formula VI



or a pharmaceutically acceptable salt thereof wherein R^{23} is alkyl of 1 to 6 carbon atoms, cycloalkyl of 3 to 7 carbon atoms, phenyl or mono- or di-substituted phenyl wherein said substituents are independently alkyl of 1 to 6 carbon atoms, alkoxy of 1 to 3 carbon atoms, halogen, or trifluoromethyl.

The present invention also provides the use of a compound of Formula VII

Formula VII



or a tautomeric form thereof and/or a pharmaceutically acceptable salt thereof, and/or a pharmaceutically acceptable solvate thereof, wherein:

A^2 represents an alkyl group, a substituted or unsubstituted aryl group, or an aralkyl group wherein the alkylene or the aryl moiety may be substituted or unsubstituted;

A^3 represents a benzene ring having in total up to 3 optional substituents;

R^{24} represents a hydrogen atom, an alkyl group, an acyl group, an aralkyl group wherein the alkyl or the aryl moiety may be substituted or unsubstituted, or a substituted or unsubstituted aryl group; or A^2 together with R^{24} represents substituted or unsubstituted C_{2-3} polymethylene group, optional substituents for the polymethylene group being selected from alkyl or aryl or adjacent substituents together with the methylene carbon atoms to which they are attached form a substituted or unsubstituted phenylene group;

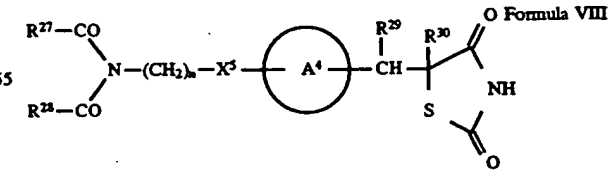
R^{25} and R^{26} each represent hydrogen, or R^{25} and R^{26} together represent a bond;

X^4 represents O or S; and

n represents an integer in the range from 2 to 6.

The present invention also provides the use of a compound of Formula VIII

Formula VIII



or a tautomeric form thereof and/or a pharmaceutically acceptable salt thereof, and/or a pharmaceutically acceptable solvate thereof, wherein:

R^{27} and R^{28} each independently represent an alkyl group, a substituted or unsubstituted aryl group, or an aralkyl group being substituted or unsubstituted in the aryl or alkyl moiety; or R^{27} together with R^{28} represents a linking group, the linking group consisting of an

optionally substituted methylene group or an O or S atom, optional substituents for the said methylene groups being selected from alkyl-, aryl, or aralkyl, or substituents of adjacent methylene groups together with the carbon atoms to which they are attached form a substituted or unsubstituted phenylene group;

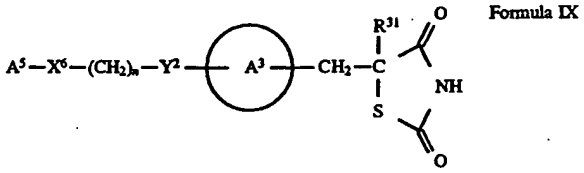
R^{29} and R^{30} each represent hydrogen, or R^{29} and R^{30} together represent a bond;

A^4 represents a benzene ring having in total up to 3 optional substituents;

X^5 represents O or S; and

n represents an integer in the range from 2 to 6.

The present invention also provides the use of a compound of Formula IX



Formula IX

or a tautomeric form thereof and/or a pharmaceutically acceptable salt thereof, and/or a pharmaceutically acceptable solvate thereof, wherein:

A^5 represents a substituted or unsubstituted aromatic heterocyclyl group;

A^6 represents a benzene ring having in total up to 5 substituents;

X^6 represents O, S, or NR^{32} wherein R^{32} represents a hydrogen atom, an alkyl group, an acyl group, an aralkyl group, wherein the aryl moiety may be substituted or unsubstituted, or a substituted or unsubstituted aryl group;

Y^2 represents O or S;

R^{31} represents an alkyl, aralkyl, or aryl group; and

n represents an integer in the range from 2 to 6.

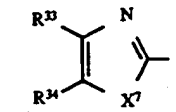
Suitable aromatic heterocyclyl groups include substituted or unsubstituted, single or fused ring aromatic heterocyclyl groups comprising up to 4 hetero atoms in each ring selected from oxygen, sulfur, or nitrogen.

Favored aromatic heterocyclyl groups include substituted or unsubstituted single ring aromatic heterocyclyl groups having 4 to 7 ring atoms, preferably 5 or 6 ring atoms.

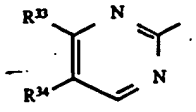
In particular, the aromatic heterocyclyl group comprises 1, 2, or 3 heteroatoms, especially 1 or 2, selected from oxygen, sulfur, or nitrogen.

Suitable values for A^5 when it represents a 5-membered aromatic heterocyclyl group include thiazolyl and oxazoyl, especially oxazoyl.

Suitable values for A^6 when it represents a 6-membered aromatic heterocyclyl group include pyridyl or pyrimidinyl.



(a)



(b)

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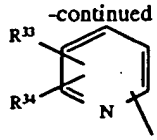


(c)

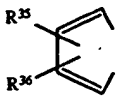
Suitable R^{31} represents an alkyl group, in particular a C_{1-6} alkyl group, for example a methyl group. Preferably, A^5 represents a moiety of formula (a), (b), or (c):

Formula (a), (b) and (c)

wherein:

R^{33} and R^{34} each independently represents a hydrogen atom, an alkyl group, or a substituted or unsubstituted aryl group or when R^{33} and R^{34} are each attached to adjacent carbon atoms, then R^{33} and R^{34} together with the carbon atoms to which they are attached form a benzene ring wherein each carbon atom represented by R^{33} and R^{34} together may be substituted or unsubstituted; and in the moiety of Formula (a), X^7 represents oxygen or sulfur.

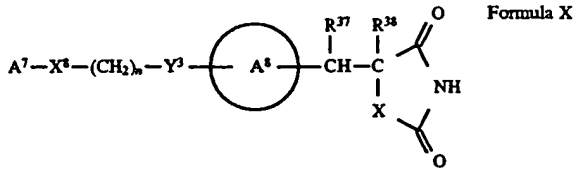
In one favored aspect R^{33} and R^{34} together represent a moiety of Formula (d):



Formula (d)

wherein R^{35} and R^{36} each independently represent hydrogen, halogen, substituted or unsubstituted alkyl, or alkoxy.

The present invention also provides for the use of compounds for Formula X



Formula X

or a tautomeric form thereof and/or a pharmaceutically acceptable salt thereof, and/or a pharmaceutically acceptable solvate thereof, wherein:

A^7 represents a substituted or unsubstituted aryl group;

A^8 represents a benzene ring having in total up to 5 substituents;

X^8 represents O, S, or NR^{39} wherein R^{39} represents a hydrogen atom, an alkyl group, an acyl group, an aralkyl group, wherein the aryl moiety may be substituted or unsubstituted, or a substituted or unsubstituted aryl group;

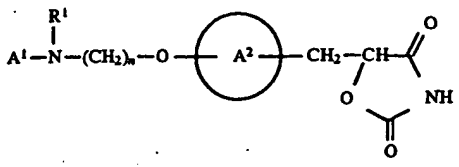
Y^3 represents O or S;

R^{37} represents hydrogen;

R^{38} represents hydrogen or an alkyl, aralkyl, or aryl group or R^{37} together with R^{38} represents a bond; and

n represents an integer in the range from 2 to 6.

The present invention is also directed to the use of compounds of Formula XI



Formula XI

or a tautomeric form thereof and/or a pharmaceutically acceptable salt thereof, and/or a pharmaceutically acceptable solvate thereof, wherein:

A^1 represents a substituted or unsubstituted aromatic heterocyclyl group;

R^1 represents a hydrogen atom, an alkyl group, an acyl group, an aralkyl group,

wherein the aryl moiety may be substituted or unsubstituted, or a substituted or unsubstituted aryl group;

A^2 represents a benzene ring having in total up to 5 substituents; and

n represents an integer in the range of from 2 to 6.

Suitable aromatic heterocyclyl groups include substituted or unsubstituted, single or fused ring aromatic heterocyclyl groups comprising up to 4 hetero atoms in each ring selected from oxygen, sulfur, or nitrogen.

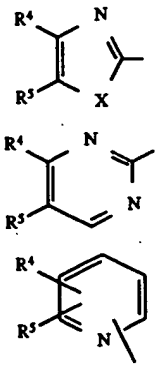
Favored aromatic heterocyclyl groups include substituted or unsubstituted single ring aromatic heterocyclyl groups having 4 to 7 ring atoms, preferably 5 or 6 ring atoms.

In particular, the aromatic heterocyclyl group comprises 1, 2, or 3 heteroatoms, especially 1 or 2, selected from oxygen, sulfur, or nitrogen.

Suitable values for A^1 when it represents a 5-membered aromatic heterocyclyl group include thiazolyl and oxazolyl, especially oxazoyl.

Suitable values for A^1 when it represents a 6-membered aromatic heterocyclyl group include pyridyl or pyrimidinyl.

Preferably, A^5 represents a moiety of formula (a), (b), or (c):



(a)

(b)

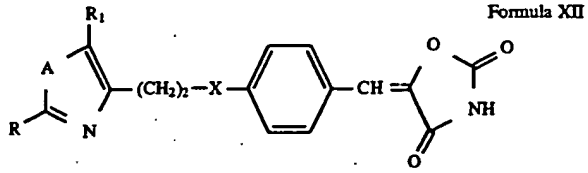
(c)

Formula (a), (b) and (c)

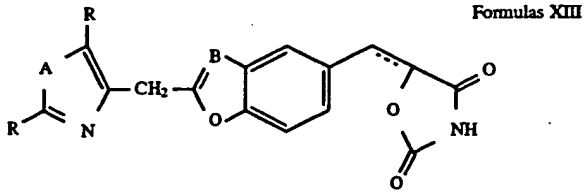
wherein:

R^4 and R^5 each independently represents a hydrogen atom, an alkyl group, or a substituted or unsubstituted aryl group or when R^4 and R^5 are each attached to adjacent carbon atoms, then R^4 and R^5 together with the carbon atoms to which they are attached form a benzene ring wherein each carbon atom represented by R^4 and R^5 together may be substituted or unsubstituted; and in the moiety of Formula (a), X represents oxygen or sulfur.

The present invention is also directed to the use of compounds of Formulas XII and XIII



Formula XII



Formulas XIII

or a pharmaceutically acceptable salt thereof wherein the dotted line represents a bond or no bond;

R is cycloalkyl of three to seven carbon atoms, naphthyl, thienyl, furyl, phenyl or substituted phenyl wherein said substituent is alkyl of one to three carbon atoms, alkoxy of one to three carbon atoms, trifluoromethyl, chloro, fluoro or bis(trifluoromethyl);

R^1 is alkyl of one to three carbon atoms;

X is O or C=O;

A is O or S; and

B is N or CH.

In one aspect the present invention is the use the compounds of Formulas I through Formula XIII, and Formulas Ia, Ib and Ic, for the treatment of the climacteric and of cancer. These compounds are herein referred to as thiazolidine derivatives. Where appropriate, the specific names of thiazolidine derivatives may be used including: Troglitazone, cioglitazone, pioglitazone and BRL 49653.

A preferred group of compounds are those of Formula XI wherein the dotted line represents no bond, R^1 is methyl, X is O and A is O. Especially preferred within this group are the compounds where R is phenyl, 2-naphthyl and 3,5-bis (trifluoromethyl)phenyl.

Another group of preferred compounds are those of Formula XII wherein the dotted line represents no bond, R^1 is methyl and A is O. Especially preferred within this group are compounds where B is CH and R is phenol, p-tolyl, m-tolyl, cyclohexyl, and 2-naphthyl. Also especially preferred is the compound where B is N and R is phenyl.

A still further embodiment of the present invention is the use of a pharmaceutical composition for administering an effective amount of a compound of the preceding Formulas I through XIII along with a pharmaceutically acceptable carrier in unit dosage form in the treatment methods mentioned above.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. Dose-response of progesterone production suppression in porcine granulosa cells treated with Troglitazone and related thiazolidinedione compounds. Porcine granulosa cells were treated for 24 h with increasing doses of Troglitazone, pioglitazone, and BRL 49653. The data represent the mean±SEM from 3 studies done in triplicate. Progesterone concentrations were corrected for DNA content in treated cells and are presented as a percentage of basal corrected progesterone production.

FIG. 2. Time course of Troglitazone suppression of progesterone production by porcine granulosa cells. Cultures of porcine granulosa cells were treated with Troglitazone, 1 and 10 μg/ml at 2,4,6,12, and 24 h. Proges-

terone production (corrected for DNA content) was measured as shown above. The data represent the mean±SEM from 3 studies done in triplicate.

FIG. 3. Cell viability of porcine granulosa cells during treatment with Troglitazone. Porcine granulosa cells were treated with various concentrations of Troglitazone and cell viability determined using a MTS dye oxidation method. This method is based on the ability of dehydrogenase enzymes found in metabolically active cells to oxidize MTS to formazan. This compound is soluble in tissue culture medium. Data represent the mean±SEM from 2 studies done in triplicate.

FIG. 4. Effects of 25-OH cholesterol on Troglitazone suppression of porcine granulosa cell progesterone production. Porcine granulosa cells were cultured for 24 h in serum-free medium (clear bars) or in the presence of 30 μg/ml of 25-OH cholesterol (shaded bars). Under both conditions, cells were treated with Troglitazone at a 1 and 10 μg/ml concentration. Data represent mean±SEM from 2 studies done in triplicate.

FIG. 5. Northern blot of Troglitazone effects on mRNA concentrations of P450 scc. Northern blot hybridization with porcine P450 scc CDNA clone of RNA (20 μg) obtained from porcine granulosa cells treated for 24 h with Troglitazone (5 μ/ml). L represents the ladder; B represents control granulosa total RNA, and T represents cell treated with Troglitazone 5 μg/ml for 24 h. Collection of total RNA and methods for Northern blot hybridization have been previously described (Urban et al., 1990).

FIG. 6. An electrophoretic mobility gel shift assay (EMSA) of nuclear extract protein from porcine granulosa treated with Troglitazone. Porcine granulosa cells were treated with Troglitazone 5 μg/ml for 24 h and nuclear extract proteins collected. Extract (15 μg) was mixed with a P^{32}-labeled consensus PPRE oligonucleotide and run on a 4% polyacrylamide gel. P is the probe alone. B is control extract. B+C is control plus 100 ×unlabeled PPRE. T is Troglitazone treatment (5 μg/ml), and T+C is Troglitazone plus 100 ×unlabeled PPRE. I is the addition of rabbit serum and A is the addition of a PPARγ antibody. The arrow indicates the supershifted PPARγ band.

FIG. 7A and 7B. Correlation of Troglitazone suppression of progesterone production with binding of PPARγ to a consensus PPRE. FIG. 7A is an autoradiogram of a EMSA from nuclear extract protein from porcine granulosa cells treated for 24 h as such: B, control cells; T, Troglitazone 5 μg/ml; BR, BRL 49635 5 μg/ml; and P, pioglitazone 5 μg/ml. The nuclear extract was mixed with radioactively-labeled consensus PPRE as described in FIG. 6. FIG. 7B is a graph of the progesterone values from the same porcine granulosa cells treated as described in A.

FIG. 8. Dose-response of Troglitazone on progesterone production by HGL5 human granulosa cell line. The human granulosa cell line HGL5 was cultured and treated with Troglitazone in a logarithmic dose-response curve as shown. Cells were treated for 48 h. Progesterone was corrected for protein concentration. The data represent the mean±SEM from 2 studies done in triplicate.

FIG. 9. Cell viability of HGL5 cells treated with Troglitazone. HGL5 cells were treated with increasing concentrations of Troglitazone for 4 days. Cell viability was assessed by the conversion of MTT to formazan as previously described. Data are the mean±SEM from 2 studies done in triplicate.

FIG. 10. EMSA of nuclear extract protein from HPL5 cells. HPL5 cells were cultured as control (B) or treated with Troglitazone (30 μg/ml) (T) for 24 h. EMSA was done with 15 μg of protein and radioactively-labeled PPRE consensus oligonucleotide. EMSA conditions are as previously described for porcine granulosa cells.

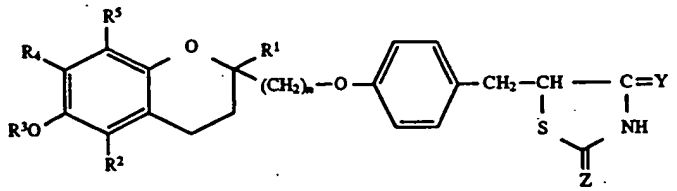
FIG. 11. Cell viability of NWTb3 cells treated with Troglitazone. NWTb3 cells were treated for 4 days with increasing concentrations of Troglitazone. Cells were grown in 24 well-plates with high-glucose MEM/10% FCS. Cell viability was assessed by the conversion of MTT to formazan as previously described. Data are the mean±SEM from 2 studies done in triplicate.

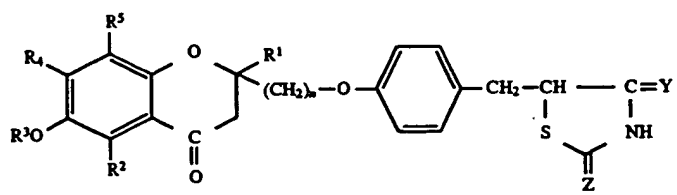
FIG. 12. EMSA of NWTb3 cells treated with Troglitazone. Nuclear extract protein (25 μg) was used in EMSA as previously described with radioactively-labeled consensus PPRE. C indicates control NWTb3 cells and T indicates NWTb3 cells treated with 10 μg/ml Troglitazone for 24 h.

FIG. 13. Cell viability of MCF-7 cells treated with Troglitazone. MCF-7 cells were treated with increasing concentrations of Troglitazone for 4 days and cell viability was assessed by the conversion of MTT to formazan as previously described. Data are the mean±SEM from 2 studies done in triplicate.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The compounds used in the treatment methods of the invention, which are 5-[4-(chromoanalkoxy)benzyl]—thiazolidene derivatives, may be represented by the Formulas (Ia), (Ib), and (Ic)





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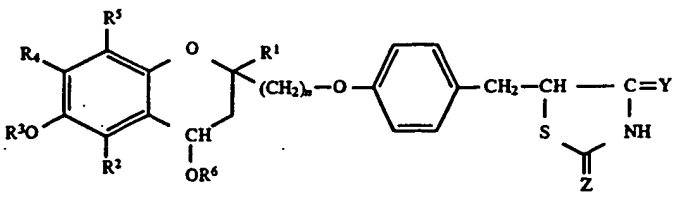


(Ic)

Formulas (Ia), (Ib), and (Ic)

(in which R^1, R^2, R^3, R^4, R^5, R^6, n, Y, and Z are as defined above) and include pharmaceutically acceptable salts thereof.

In the compounds of the invention where R^1 or R^2 represents an alkyl group, this may be a straight or branched chain alkyl group having from 1 to 5 carbon atoms and is preferably a primary or secondary alkyl group, for example, the methyl, ethyl, propyl, isopropyl, butyl, isobutyl, pentyl, or isopentyl group.

Where R^3, R^6, or $R^{6'}$ represents an aliphatic acyl group, this preferably has from 1 to 6 carbon atoms and may include one or more carbon-carbon double or triple bonds. Examples of such groups include the formyl, acetyl, propionyl, butyryl, isobutyryl, pivaloyl, hexanoyl, acryloyl, methacryloyl, and crotonyl groups.

Where R^3, R^6, or $R^{6'}$ represents an alicyclic acyl group, it is preferably a cyclopentanecarbonyl, cyclohexanecarbonyl, or cycloheptanecarbonyl group.

Where R^1, R^6, or $R^{6'}$ represents an aromatic acyl group, the aromatic moiety thereof may optionally have one or more substituents (for example, nitro, amino, alkylamino, dialkylamino, alkoxy, halo, alkyl, or hydroxy substituents); examples of such aromatic acyl groups included the benzoyl, p-nitrobenzoyl, m-fluorobenzoyl, o-chlorobenzoyl, p-aminobenzoyl, m-(dimethylamino)benzoyl, o-methoxybenzoyl, 3,4-dichlorobenzoyl, 3,5-di-t-butyl-4-hydroxybenzoyl, and 1-naphthoyl groups.

Where R^3, R^6, or $R^{6'}$ represents a heterocyclic acyl group, the heterocyclic moiety thereof preferably has one or more, preferably one, oxygen, sulfur, or nitrogen hetero atoms and has from 4 to 7 ring atoms; examples of such heterocyclic acyl groups include the 2-furoyl, 3-thenoyl, 3-pyridinecarbonyl (nicotinoyl), and 4-pyridinocarbonyl groups.

Where R^3, R^6, or $R^{6'}$ represents an araliphatic acyl group, the aliphatic moiety thereof may optionally have one or more carbon-carbon double or triple bonds and the aryl moiety thereof may optionally have one or more substituents (for example, nitro, amino, alkylamino, dialkylamino, alkoxy, halo, alkyl, or hydroxy substituents); examples of such araliphatic acyl groups include the phenylacetyl, p-chlorophenylacetyl, phenylpropionyl, and cinnamoyl groups.

Where R^3, R^6, or $R^{6'}$ represents a (C_1–C_6 alkoxy)carbonyl group, the alkyl moiety thereof may be any one of those alkyl groups as defined for R^1 and R^2, but is preferably a methyl or ethyl group, and the alkoxycarbonyl group represented by R^3, R^6, or $R^{6'}$ is therefore preferably a methoxycarboniyl or ethoxycarbonyl group.

Where R^3, R^6, or $R^{6'}$ represents an aralkyloxycarbonyl group, the aralkyl moiety thereof may be any one of those included within the araliphatic acyl group represented by R^3, R^6, or $R^{6'}$, but is preferably a benzyloxycarbonyl group.

Where R^4 and R^5 represent alkyl groups, they may be the same or different and may be straight or branched chain alkyl groups. They preferably have from 1 to 5 carbon atoms and examples include the methyl, ethyl, propyl, isopropyl, butyl, isobutyl, t-butyl, pentyl, and isopentyl groups.

Where R^4 and R^5 represent alkoxy groups, these may be the same or different and may be straight or branched chain groups, preferably having from 1 to 4 carbon atoms. Examples include the methoxy, ethoxy, propoxy, isopropoxy, and butoxy groups. Alternatively, R^4 and R^5 may together represent a C_1–C_4 alkylenedioxy group, more preferably a methylenedioxy or ethylenedioxy group.

Preferred classes of compounds of Formula I are as follows:

(1) Compounds in which R^3 represents a hydrogen atom, a C_1–C_6 aliphatic acyl group, an aromatic acyl group, or a heterocyclic acyl group.

(2) Compounds in which Y represents an oxygen atom; R^1 and R^2 are the same or different and each represents a hydrogen atom or a C_1–C_5 alkyl group; R^3 represents a hydrogen atom, a C_1–C_6 aliphatic acyl group, an aromatic acyl group, or a pyridinecarbonyl group; and R^4 and R^5 are the same or different and each represents a hydrogen atom, a C_1–C_5 alkyl group, or a C_1 or C_2 alkoxy group.

(3) Compounds as defined in (2) above, in which R^1, R^2, R^4 and R^5 are the same or different and each represents a hydrogen atom or a C_1–C_5 alkyl group; n is 1 or 2; and W represents the —CH_2— or >CO group.

(4) Compounds as defined in (3) above, in which R^3 represents a hydrogen atom, a C_1–C_5 aliphatic acyl group, a benzoyl group, or a nicotinyl group.

(5) Compounds as defined in (4) above, in which: R^1 and R^4 are the same or different and each represents a C_1–C_5 alkyl group; R^2 and R^5 are the same or different and each represents the hydrogen atom or the methyl group; and R^3 represents a hydrogen atom or a C_1–C_4 aliphatic acyl group.

(6) Compounds in which: W represents the —CH_2— or >CO group; Y and Z both represent oxygen atoms; n is 1 or 2; R^1 and R^4 are the same or different and each represents a C_1–C_4 alkyl group; R^2 and R^5 are the same or different and each represents the hydrogen atom or the methyl group; and R^3 represents a hydrogen atom or a C_1–C_4 aliphatic acyl group.

(7) Compounds as defined in (6) above, in which n is 1.

(8) Compounds as defined in (6) or (7) above, in which W represents the —CH_2— group. Preferred compounds among the compounds of Formula I are those wherein:

- R^1 is a C1–C4 alkyl group, more preferably a methyl or isobutyl group, most preferably a methyl group;
- R^2 is a hydrogen atom or a C_1–C_4 alkyl group, preferably a hydrogen atom, or a methyl or isopropyl group, more preferably a hydrogen atom or a methyl group, most preferably a methyl group;
- R^3 is a hydrogen atom, a C_1–C_4 aliphatic acyl group, an aromatic acyl group or a pyridinecarbonyl group, preferably a hydrogen atom, or an acetyl, butyryl, benzoyl, or nicotinyl group, more preferably a hydrogen atom or an acetyl, butyryl or benzoyl group, most preferably a hydrogen atom or an acetyl group;

R^4 is a hydrogen atom, a C_1–C_4 alkyl group or a C_1 or C_2 alkoxy group, preferably a methyl, isopropyl, t-butyl, or methoxy group, more preferably a methyl or t-butyl group, most preferably a methyl group;

R^5 is a hydrogen atom, a C_1–C_4 alkyl group or a C_1 or C_2 alkoxy group, preferably a hydrogen atom, or a methyl or methoxy group, more preferably a hydrogen atom or a methyl group, and most preferably a methyl group;

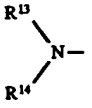
n is 1 or 2, preferably 1;

Y is an oxygen atom;

Z is an oxygen atom or an imino group, most preferably an oxygen atom; and

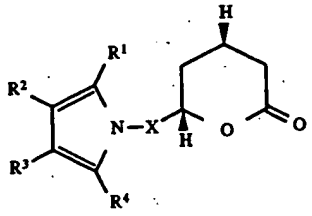
W is a —CH_2— or >C=O group, preferably a —CH_2 group.

Referring to the general Formula II, the substituents may be any from 1 to 3 selected from nitro, amino, alkylamino, dialkylamino, alkoxy, halo, alkyl, or hydroxy, the aromatic acyl group may be benzoyl and naphthoyl. The alkyl group R^{11} may be a straight chain or branched alkyl of 1 to 10 carbon atoms, such as methyl, ethyl, n-propyl, i-propyl, n-butyl, i-butyl, t-butyl, n-pentyl, i-pentyl, n-hexyl, n-heptyl, n-octyl, n-nonyl, and n-decyl; the cycloalkyl group R^{11} may be a cycloalkyl group of 3 to 7 carbon atoms, such as cyclopropyl, cyclopentyl, cyclohexyl, and cycloheptyl and the phenylalkyl group R^{11} may be a phenylakyl group of 7 to 11 carbon atoms such as benzyl and phenethyl. As examples of the heterocyclic group R^{11} may be mentioned 5- or 6-membered groups each including 1 or 2 hetero-atoms selected from among nitrogen, oxygen, and sulfur, such as pyridyl, thienyl, furyl, thiazolyl, etc. When R^{11} is



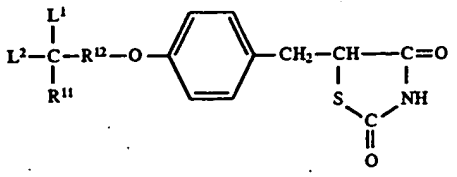
Possible Formula Used for R^{11}

the lower alkyls R^{13} and R^{14} may each be a lower alkyl of 1 to 4 carbon atoms, such as methyl, ethyl, n-propyl, i-propyl, and n-butyl. When R^{13} and R^{14} are combined to each other to form a 5- or 6-membered heterocyclic group as taken together with the adjacent N atom, i.e., in the form of



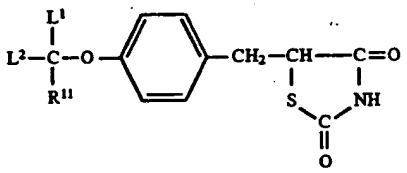
R^{13} and R^{14} are combined to each other to form a 5- or 6-membered heterocyclic group

this heterocyclic group may further include a heteroatom selected from among nitrogen, oxygen, and sulfur as exemplified by piperidino, morpholino, pyrrolidino, and piperazino. The lower alkylene group R^{12} may contain 1 to 3 carbon atoms and thus may be, for example, methylene, ethylene, or trimethylene. The bond R^{12} is equivalent to the symbol "-", ".", or the like which is used in chemical structural formulas, and when R^{12} represents such a bond, the compound of general Formula II is represented by the following general Formula II(a)



Formula II(a)

Thus, when R^{12} is a bond, the atoms adjacent thereto on both sides are directly combined together. As examples of the lower alkyls L^1 and L^2, there may be mentioned lower alkyl groups of 1 to 3 carbon atoms, such as methyl and ethyl. The alkylene group formed as L^1 and L^2 are joined together is a group of the formula —$(CH_2)_n$— [where n is an integer of 2 to 6]. The cycloalkyl, phenylalkyl, phenyl, and heterocyclic groups mentioned above, as well as said heterocyclic group



Heterocyclic Group

may have 1 to 3 substituents in optional positions on the respective rings. As examples of such substituents may be mentioned lower alkyls (e.g., methyl, ethyl, etc.), lower alkoxy groups (e.g., methoxy, ethoxy, etc.), halogens (e.g., chlorine, bromine, etc.), and hydroxyl. The case also falls within the scope of the general Formula II that an alkylenedioxy group of the formula —O—$(CH_2)_m$—O— [is an integer of 1 to 3], such as methylenedioxy, is attached to the two adjacent carbon atoms on the ring to form an additional ring.

The preferred compounds of Formula III are those wherein R^{15} and R^{16} are independently hydrogen, lower alkyl containing 1 to 6 carbon atoms, alkoxy containing 1 to 6 carbon atoms, halogen, ethynyl, nitrile, trifluoromethyl, vinyl, or nitro; n is 1 or 2 and the pharmaceutically acceptable salts thereof.

Preferred in Formula IV are compounds wherein the dotted line represents no bond, particularly wherein D is CO or CHOH. More preferred are compounds wherein V is —CH=CH—, —CH=N— or S and n is 2, particularly those compounds wherein X is O and Y is N, X is S and Y is N, X is S and Y is CH or X is —H=N— and Y is CH. In the most preferred compounds X is O or S and Y is N forming an oxazol-4-yl, oxazol-5-yl, thiazol-4-yl, or thiazol-5-yl group; most particularly a 2-[(2-thienyl), (2-furyl), phenyl, or substituted phenyl]-5-methyl-4-oxazolyl group.

The preferred compounds in Formula V are:

a) those wherein the dotted line represents no bond, A and B are each CH, X^1, is CO, n is O, R^{19} is hydrogen, Z^2 is CH_2CH_2 or CH=CH and X^3 is hydrogen, particularly when X^2 is hydrogen, 2-methoxy, 4-benzyloxy, or 4-phenyl;

b) those wherein A and B are each CH, X^1 is S or SO_2, n is O, R^{19} is hydrogen, Z^2 is CH_2CH_2 and X^3 is hydrogen, particularly when X^2 is hydrogen or 4-chloro.

A preferred group of compounds is that of Formula VI wherein R^{23} is (C_1–C_6)alkyl, (C_3–C_7)cycloalkyl, phenyl, halophenyl, or (C_1–C_6—)alkylphenyl. Especially preferred within this group are the compounds where R^{23} is phenyl, methylphenyl, fluorophenyl, chlorophenyl, or cyclohexyl.

When used herein with regard to Formulas VII through X, the term "aryl" includes phenyl and naphthyl, suitably

phenyl, optionally substituted with up to 5, preferably up to 3, groups selected from halogen, alkyl, phenyl, alkoxy, haloalkyl, hydroxy, amino, nitro, carboxy, alkoxycarbonyl, alkoxycarbonylalkyl, alkylcarbonyloxy, or alkylcarbonyl groups.

The term "halogen" refers to fluorine, chlorine, bromine, and iodine; preferably chlorine.

The terms "alkyl" and "alkoxy" relate to groups having straight or branched carbon chains, containing up to 12 carbon atoms.

Suitable alkyl groups are C_{1-12} alkyl groups, especially C_{1-6} alkyl groups, e.g., methyl, ethyl, n-propyl, iso-propyl, n-butyl, isobutyl, or tert-butyl groups.

Suitable substituents for any alkyl group include those indicated above in relation to the term "aryl".

Suitable substituents for any heterocyclyl group include up to 4 substituents selected from the group consisting of alkyl, alkoxy, aryl, and halogen or any 2 substituents on adjacent carbon atoms, together with the carbon atoms to which they are attached, may form an aryl group, preferably a benzene ring, and wherein the carbon atoms of the aryl group represented by the said 2 substituents may themselves be substituted or unsubstituted.

Specific examples of compounds of the present invention are given in the following list:

(+)-5-[[4-[(3 ,4-dihydro-6-hydroxy-2,5,7,8-tetramethyl-2H-1-benzopyran-2-yl)methoxy]phenyl]methyl]-2,4-thiazolidinedione: (Troglitazone);

5-[4-[2-(5-ethylpyridin-2-yl)ethoxyl]benzyl] thiadiazolidine-2,4-dione: (pioglitazone);

5-[4-[(1-methylcyclohexyl)methoxy]benzyl] thiadiazolidine-2,4-dione: (ciglitazone);

4-(2-naphthylmethyl)- 1,2,3,5-oxathiadiazole-2-oxide;

5-[4-[2-[N-(benzoxazol-2-yl)-N-methylamino]ethoxy] benzyl]-5-methylthiazolidine-2,4-dione;

5-[4-[2- [2,4-dioxo-5-phenylthiazolidin-3-yl)ethoxy] benzyl]thiazolidine-2,4-dione;

5-[4-[2-[N-methyl-N-(phenoxycarbonyl)amino]ethoxy] benzyl]thiazolidine-2,4-dione;

5-[4-[2-phenoxyethoxy)benzyl]thiazolidine-2,4-dione;

5-[4-[2-(4-chlorophenyl)ethylsulfonyl]benzyl]thiazolidine-2,4-dione;

5-[4-[3-(5-methyl-2-phenyloxazol-4-yl)propionyl]benzyl] thiazolidine-2,4-dione;

5-[[4-(3-hydroxy-1-methylcyclohexyl)methoxy]benzyl] thiadiazolidine-2,4-dione;

5-[4-[2-(5-methyl-2-phenyloxazol-4-yl)ethoxyl]benzyl] thiadizolidione-2,4-dione;

5-[(2-benzyl-2,3-dihydrobenzopyran)-5-ylmethyl] thiadiazoline-2,4-dione: (englitazone);

5-[[2-(2-naphthylmethyl)benzoxazol]-5-ylmethyl] thiadiazoline-2,4-dione;

5-[4-[2-(3-phenylureido)ethoxyl]benzyl]thiadiazoline-2,4-dione;

5-[4-[2-[N-(benzoxazol-2-yl)-N-methylaiino]ethoxy]benzy] thiadiazoline-2,4-dione;

5-[4-[3-(5-methyl-2-phenyloxazol-4-yl)propionyl]benzyl] thiadiazoline-2,4-dione;

5-[2-(5-methyl-2-phenyloxazol-4-ylmethyl)benzofuran-5-ylmethyl]-oxazolidine-2,4-dione;

5-[4-[2-[N-methyl-N-(2-pyridyl)amino]ethoxy]benzyl] thiazolidine-2,4-dione (BRL 49653); and

5-[4-[2-[N-(benzoxazol-2-yl)-N-methylamino]ethoxy] benzyl]-oxazolidine-2,4-dione.

The compounds of Formulas I through XIII are capable of further forming pharmaceutically acceptable acid addition and/or base salts.

The compounds of Formulas I through XIII are capable of further forming both pharmaceutically acceptable acid addition and/or base salts. All of these forms are within the scope of the present invention.

Pharmaceutically acceptable acid addition salts of the compounds of Formulas I through XIII include salts derived from nontoxic inorganic acids such as hydrochloric, nitric, phosphoric, sulfuric, hydrobromic, hydriodic, hydrofluoric, phosphorous, and the like, as well as the salts derived from nontoxic organic acids, such as aliphatic mono- and dicarboxylic acids, phenyl-substituted alkanoic acids, hydroxy alkanoic acids, alkanedioic acids, aromatic acids, aliphatic and aromatic sulfonic acids, etc. Such salts thus include sulfate, pyrosulfate, bisulfate, sulfite, bisulfite, nitrate, phosphate, monohydrogenphosphate, dihydrogenphosphate, metaphosphate, pyrophosphate, chloride, bromide, iodide, acetate, trifluoracetate, propionate, caprylate, isobutyrate, oxalate, malonate, succinate, suberate, sebacate, fumarate, malcate, mandelate, benzoate, chlorobenzoate, methylbenzoate, dinitrobenzoate, phthalate, benzenesulfonate, toluenesulfonate, phenylacetate, citrate, lactate, maleate, tartrate, methanesulfonate, and the like. Also contemplated are salts of amino acids such as arginate and the like and gluconate, galacturonate, n-methyl glucamine (see, for example, Berge et al., 1977).

The acid addition salts of said basic compounds are prepared by contacting the free base form with a sufficient amount of the desired acid to produce the salt in the conventional manner. The free base form may be regenerated by contacting the salt form with a base and isolating the free base in the conventional manner or as above. The free base forms differ from their respective salt forms somewhat in certain physical properties such as solubility in polar solvents, but otherwise the salts are equivalent to their respective free base for purposes of the present invention.

Pharmaceutically acceptable base addition salts are formed with metals or amines, such as alkali and alkaline earth metals or organic amines. Examples of metals used as cations are sodium, potassium, magnesium, calcium, and the like. Examples of suitable amines are N_2N'-dibenzylethylenediamine, chloroprocaine, choline, diethanolamine, dicyclohexylamine, ethylenediamine, N-methylglucamine, and procaine (see, for example, Berge et al., 1977).

The base addition salts of said acidic compounds are prepared by contacting the free acid form with a sufficient amount of the desired base to produce the salt in the conventional manner. The free acid form may be regenerated by contacting the salt form with an acid and isolating the free acid in the conventional manner or as above. The free acid forms differ from their respective salt forms somewhat in certain physical properties such as solubility in polar solvents, but otherwise the salts are equivalent to their respective free acid for purposes of the present invention.

Certain of the compounds of the present invention can exist in unsolvated forms as well as solvated forms, including hydrated forms. In general, the solvated forms, including hydrated forms, are equivalent to unsolvated forms and are intended to be encompassed within the scope of the present invention. Certain of the compounds of the present invention possess one or more chiral centers and each center may exist in different configurations. The compounds can, therefore, form stereoisomers. Although these are all represented herein by a limited number of molecular formulas, the present invention includes the use of both the individual, isolated isomers and mixtures, including racemates, thereof. Where stereospecific synthesis techniques are employed or

optically active compounds are employed as starting materials in the preparation of the compounds, individual isomers may be prepared directly; on the other hand, if a mixture of isomers is prepared, the individual isomers may be obtained by conventional resolution techniques, or the mixture may be used as is, without resolution.

Furthermore, the thiazolidene or oxazlidene part of the compounds of Formulas I through XIII can exist in the form of tautomeric isomers. All of the tautomers are represented by Formulas I through XIII, and are intended to be a part of the present invention.

For preparing pharmaceutical compositions from the compounds of the present invention, pharmaceutically acceptable carriers can be either solid or liquid. Solid form preparations include powders, tablets, pills, capsules, cachets, suppositories, and dispersible granules. A solid carrier can be one or more substances which may also act as diluents, flavoring agents, binders, preservatives, tablet disintegrating agents, or an encapsulating material.

In powders, the carrier is a finely divided solid which is in a mixture with the finely divided active component.

In tablets, the active component is mixed with the carrier having the necessary binding properties in suitable proportions and compacted in the size and shape desired.

The powders and tablets preferably contain from five or ten to about seventy percent of the active compound. Suitable carriers are magnesium carbonate, magnesium stearate, talc, sugar, lactose, pectin, dextrin, starch, gelatin, tragacanth, methylcellulose, sodium carboxymethylcellulose, a low melting wax, cocoa butter and the like. The term "preparation" is intended to include the formulation of the active compound with encapsulating material as a carrier providing a capsule in which the active component with or without other carriers, is surrounded by a carrier, which is thus in association with it. Similarly, cachets and lozenges are included. Tablets, powders, capsules, pills, cachets, and lozenges can be used as solid dosage forms suitable for oral administration.

For preparing suppositories, a low melting wax, such as a mixture of fatty acid glycerides or cocoa butter, is first melted and the active compound is dispersed homogeneously therein, as by stirring. The molten homogenous mixture is then poured into convenient sized molds, allowed to cool, and thereby to solidify.

Liquid form preparations include solutions, suspensions, and emulsions, for example, water or water proylene glycol solutions. For parenteral injection liquid preparations can be formulated in solution in aqueous polyethylene glycol solution.

Aqueous solutions suitable for oral use can be prepared by dissolving the active component in water and adding suitable colorants, flavors, stabilizing and thickening agents as desired.

Aqueous suspensions suitable for oral use can be made by dispersing the finely divided active component in water with viscous material, such as natural or synthetic gums, resins, methylcellulose, sodium carboxymethylcellulose, and other well-known suspending agents.

Also included are solid form preparations which are intended to be converted, shortly before use, to liquid form preparations for oral administration. Such liquid forms include solutions, suspensions, and emulsions. These preparations may contain, in addition to the active component, colorants, flavors, stabilizers, buffers, artificial and natural sweeteners, dispersants, thickeners, solubilizing agents, and the like.

The pharmaceutical preparation is preferably in unit dosage form. In such form the preparation is subdivided into unit doses containing appropriate quantities of the active component. The unit dosage form can be a packaged preparation, the package containing discrete quantities of preparation, such as packeted tablets, capsules, and powders in vials or ampoules. Also, the unit dosage form can be a capsule, tablet, cachet, or lozenge itself, or it can be the appropriate number of any of these in packaged form.

The quantity of active component in a unit dose preparation may be varied or adjusted from 0.1 mg to 100 mg preferably 0.5 mg to 100 mg according to the particular application and the potency of the active component. The composition can, if desired, also contain other compatible therapeutic agents.

The compounds of Formulas I through XIII are valuable agents for the treatment of the climacteric and of cancer. The following illustrates testing to show that compounds have the disclosed activity, using the preferred compound Troglitazone and related thiazolidinedione derivatives.

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1

Suppression of Progesterone Production in Porcine Granulosa Cells with Troglitazone and Related Compounds

Initial studies investigated the dose-response of Troglitazone and related compounds on progesterone production in cultures of porcine granulosa cells. Porcine granulosa cells were isolated from 1- to 5-mm follicles of ovaries from immature swine (60–70 kg) obtained from a local slaughterhouse (Urban et al., 1994). The cells were plated in Eagle's minimum essential medium and 3% fetal calf serum (FCS) at a concentration of 20–30 million cells/60-mm dish for 12–16 h to facilitate granulosa cell attachment to the plates. Monolayer cultures were maintained at 37° C. in 5% CO_2 throughout the study. After granulosa cell attachment, medium containing the FCS was discarded and serum-free medium with varying concentrations of Troglitazone, pioglitazone and BRL 49653 was added for 24 h. One ml of medium was collected for measurement of progesterone by an assay previously described that uses the chromatographic separation of steroids to enhance specificity (Urban et al., 1994). As shown in FIG. 1, all three of the drugs suppressed progesterone production by porcine granulosa cells in a dose-dependent fashion, but Troglitazone was more potent at suppressing progesterone production than 2 other drugs in the thiazolidinedione class of drugs (FIG. 1). This demonstrated the likely utility of Troglitazone and related thiazolidinedione derivatives in the treatment of the climacteric, and that thiazolidine derivatives have physiologic activities similar to those of Troglitazone.

The time course for the suppression of progesterone was done in the same porcine granulosa cell culture system. Cells were treated for up to 24 h with zero, 1, or 10 μg/ml of Troglitazone. As shown in FIG. 2, progesterone production was suppressed by Troglitazone as early as 4 h. The concentration of Troglitazone did not affect the time of onset of suppression (FIG. 2).

EXAMPLE 2

Cell Viability of Porcine Granulosa Cells During Treatment With Troglitazone

Although there was not a significant decrease in DNA content between control and Troglitazone-treated porcine granulosa cells, total protein concentrations were lower in the Troglitazone treatment groups. Therefore, cell viability was tested in these cells using a colorimetric assay that is dependent on the oxidation of MTS to formazan (absorbance measured at 490 nm) by dehydrogenase enzymes found in metabolically active cells (Promega, Madison Wis.). This assay was verified in porcine granulosa cells by showing a linear increase in absorption with increasing cell density. Even at the highest doses of Troglitazone (100 μg/ml), there was not a decrease in cell viability in granulosa cells (FIG. 3). This demonstrates that Troglitazone and related thiazolidinedione derivatives are unlikely to affect the viability of normal granulosa cells.

EXAMPLE 3

Studies on the Mechanism of Troglitazone Suppression of Porcine Granulosa Cell Progesterone Production

The suppression of progesterone production in porcine granulosa cells by Troglitazone could be due to effects of the drug on the supply of cholesterol to the mitochondria. Before investigating proteins in the pathway of cholesterol transport to the mitochondria, it was first tested whether Troglitazone would inhibit progesterone production when cells were co-treated with 25-OH cholesterol. This compound diffuses directly to the P-450 cholesterol side-chain cleavage enzyme (P450 scc) in the mitochondria and acts as a substrate for progesterone production (Veldhuis and Furlanetto, 1985). Treatment of porcine granulosa cells with Troglitazone and 25-OH cholesterol demonstrated that even in the presence of 25-OH cholesterol, Troglitazone suppressed progesterone production (FIG. 4).

Having shown that Troglitazone did not impair the cholesterol transport system in porcine granulosa cells, the effects of the drug on the mRNA concentrations of the rate-limiting enzyme in the steroidogenic pathway, P450 scc, were determined (Miller, 1988). Suppression of transcription of this enzyme would result in decreased progesterone production by granulosa cells. Porcine granulosa cells were cultured and treated with Troglitazone (5 μg/ml) for 24 h. Troglitazone treatment did not suppress mRNA concentrations of P450 scc (FIG. 5) suggesting that the compound does not inhibit progesterone synthesis by decreasing expression of this specific enzyme.

With no evidence of a direct effect of Troglitazone on the transport of cholesterol or the mRNA concentrations of the rate-limiting enzyme for steroidogenesis, P450 scc, a more general effect of Troglitazone on cellular function was studied. As stated above, Troglitazone is a ligand for the PPARγ orphan nuclear receptor (Lehmann, 1995). This receptor binds to a DR-1 consensus element that is non-specific and will bind many transcription factors in this nuclear receptor family. It was indicated that a marked translocation of PPARγ into the nucleus could disrupt the function of many genes. This mechanism would make rapidly growing cells expressing PPARγ, such as those of many types mesenchymal tumors, more susceptible to this phenomenon. A DR-1 consensus oligonucleotide was synthesized (NNN-AGGTCA-N-AGGTCA, SEQ ID NO:1) and used in electrophoretic mobility gel shift assay (EMSA) with 15 μg of nuclear extract protein from porcine granulosa cells treated with Troglitazone, pioglitazone and BRL 49653. All three of the compounds, Troglitazone, pioglitazone and BRL 49653 increased binding to the PPRE in granulosa cell nuclear extracts (FIG. 6), indicating that all three compounds had a similar effect with respect to binding of the DR-1 consensus sequence, although the binding was greatest with Troglitazone. Binding was specific in that unlabeled PPRE competitively inhibited binding of the labeled DNA (FIG. 6). Moreover, an antibody to PPARγ showed a supershifted band in EMSA (FIG. 6).

To further correlate the enhanced binding of PPARγ to the DR-1 consensus sequence after treatment with Troglitazone, cultures of porcine granulosa cells were treated with Troglitazone, pioglitazone, and BRL 49653 (all at a 5 μg/ml concentration) and nuclear extract protein was collected. As shown in FIG. 7A and FIG. 7B, suppression of progesterone in these cells occurred with Troglitazone and this correlated with the increased binding to the DR-1 consensus sequence in EMSA.

EXAMPLE 4

Dose-Response of Human HGL5 Cells Treated with Troglitazone

A PPARγ expressing human granulosa cell line (HGL5) was selected that had been transformed by the E6 and E7 regions of the human papillomavirus (Rainey el al., 1994). Although these cells were slow growing in cell culture, they retained their steroidogenic capacity (Rainey et al., 1994). Troglitazone also suppressed progesterone production in this cell line, although the suppression occurred only at concentrations of Troglitazone greater than 10 μg/ml (FIG. 8). Moreover, there was a biphasic response to Troglitazone, with lower concentrations actually increasing progesterone production (FIG. 8).

EMSA was next performed on nuclear extract protein from HGL5 cells to determine whether the increase in binding to the DR-1 consensus sequence induced by Troglitazone in porcine granulosa cells was also present in this cell line. Troglitazone at a 30 μg/ml concentration inhibited progesterone production by these cells and also markedly increased binding to the PPRE (FIG. 10).

The effects of Troglitazone on cell viability in the HGL5 cells was next tested. A similar assay was used as described above for porcine granulosa cells, using MTT instead of MTS for conversion to formazan. As shown in FIG. 9, Troglitazone concentrations as low as 0.5 μg/ml caused a loss of cell viability. This demonstrated the utility of Troglitazone in decreasing the viability of this immortal, transformed cell line.

EXAMPLE 5

Treatment of Mouse Fibroblast NWTb3 Cells with Troglitazone: Effects on Cell Viability, and Binding to the PPRE

Because a decrease in cell viability was observed with the human granulosa cell line (HGL5), the effects of Troglitazone on 2 other cell lines was studied. The first cell line tested with Troglitazone was a PPARγ expressing transformed mouse fibroblast line derived from NIH 3T3 cells which over expresses the IGF-I receptor (NWTb3). These cells are of mesenchymal origin and grow more rapidly than the HGL5 line. In this cell line Troglitazone caused a loss of

cell viability at concentrations of 1.0 μg/ml and greater (FIG. 11). These results indicated that Troglitazone is effective in decreasing the viability of PPARγ-expressing transformed cell lines, and further indicate the applicability of Troglitazone in the treatment of tumor cells.

EMSA was also done on nuclear extract protein from NWTb3 cells treated with 10 μg/ml of Troglitazone to determine whether an increase in binding to the PPRE was seen as in the 2 previous systems (porcine and human granulosa). As shown in FIG. 12, an increase in binding of PPRE was seen during treatment with Troglitazone. This again, suggests that the effects of troglitazone and related thiazolidinedione derivatives are mediated by binding to the DR-1 consensus sequence.

EXAMPLE 6

Effects of Troglitazone on the Viability of Human MCF-7 Cells

A non-PPARγ expressing human breast cancer cell line, MCF-7, was tested. In this cell line, which is non-mesenchymal in origin, cell viability decreased only with high concentrations of Troglitazone (FIG. 13). Nuclear extract protein collected from MCF-7 cells treated with 30 μg/ml of Troglitazone for 24 h and used in EMSA with the DR-1 consensus sequence showed no presence of binding. The results show that loss of cell viability is correlated with the expression of PPARγ in transformed and cancer cells.

EXAMPLE 7

Treatment of the Climacteric with Troglitazone

A female human patient typically enters the climacteric from about age 40 to age 55, resulting in irregular menses and episodes of prolonged bleeding. In the case of a patient with a history of deep venous thrombosis, severe hypertension, severe hyperlipidemia, breast cancer, endometrial cancer, or cholelithiasis, Troglitazone would be the primary treatment option. In the absence of these risk factors, Troglitazone may be offered as an option to hormone treatment. Prevention of irregular menstrual bleeding may be achieved in a human climacteric patient by administration of a therapeutically active amount of Troglitazone. During an initial treatment period, administration of Troglitazone in an amount of about 200 mg per day may be directed with monitoring of condition. If symptoms continue, administration may be increased to about 400 mg per day. Estrogen levels in the patient can be periodically monitored for low estrogen levels or symptoms of low estrogen levels, such as hot flashes. Treatment with Troglitazone may continue until these symptoms develop or there is evidence that the patient is menopausal. Such evidence may be suggested by an elevation in levels of follicle stimulating hormone.

EXAMPLE 8

Treatment of a Human Cancer Patient with Troglitazone

Troglitazone may be used for the treatment of tumor cells expressing PPARγ. The studies documented herein, demonstrate that Troglitazone can be used to reduce the viability of PPARγ-expressing cell lines, and that rapidly growing cells are particularly sensitive to Troglitazone. Thus tumors that are known to express PPARγ would be tumor types likely to be treated with troglitazone. Mesenchymal tumors are one type of tumor which is particularly likely to express PPARγ.

In cases where it is not known if a tumor expresses PPARγ, one may use a number of methods to screen tumor cells for expression of PPARγ including EMSA, as well as Northern and Western Blot analysis. Tumor cell lines may be also be screened for sensitivity to Troglitazone in vitro or in vivo. Cultured tumor cells may be treated with concentrations of Troglitazone varying from about 0.1 μg to about 30 μg. Viability of the tumors cells may then be assayed using a colorimetric assay that is dependent on the oxidation of MTS to formazan (absorbance measured at 490 nm) by dehydrogenase enzymes found in metabolically active cells (Promega, Madison Wis.). In vivo treatment of a human cancer patient with Troglitazone may be achieved by administration of about 400 mg per day of Troglitazone. An initial period of treatment with Troglitazone or a thiazolidinedione derivative of a cancer patient may be initiated with monitoring of condition. Treatment may be continued or discontinued based on condition of the patient. Use of Troglitazone therapy in conjunction with other chemotherapeutic agents, radiation, or surgery may in many cases be the preferred mode of treatment. Troglitazone treatment therefore, would inhibit the growth of the cancer so that other therapies may be added, thereby increasing the likelihood of curing the patient. Troglitazone and related thiazolidinedione derivatives may additionally be used to treat patients with severely metastatic disease. Such treatment may slow tumor growth and reduce tumor mass, thereby prolonging survival and increasing the quality of life of terminal cancer patients.

REFERENCES

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

Barentsen R. Eur J Obs Gyn & Repr Biol 64 Suppl:s7–11. 1996

Berge et al., "Pharmaceutical Salts," *J Pharm. Science,* 66:1–19. 1977.

Connelly E F. Budd G T. Seminars in Oncology 23:16–21. 1996

Dunaif, Scott, Finegood, Quintana, Whitcomb, "The insulin-sensitizing agent Troglitazone improves metabolic and reproductive abnormalities in the polycystic ovary syndrome ." *J. Clin. Endocrinol. Metab.,* 81:3299–3306. 1996.

Forman, Tontonoz, Chen, Brun, Spiegelman, Evans, "15-Deoxy-delta^{12,14}-Prostaglandin J_2 is a ligand for the adipocyte determination factor PPAR gamma," *Cell,* 83:803–812. 1995.

Kliewer, Lenhard, Wilson, Patel, Morris, Lehman, "A prostaglandin J_2 metabolite binds peroxisome proliferator-activated receptor gamma and promotes adipocyte differentiation," *Cell,* 83:813–819. 1995.

Lehmann, Moore, Smith-Oliver, Wilkison, Willson, Kliewer, "An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor gamma (PPARgamma)," *J. Biol. Chem.,* 270:12953–12956. 1995.

Marcus, Capone, Rachubinski, "Identification of COUP-TFII as a peroxisome proliferator response element binding factor using genetic selection in yeast: COUP-TFII activates transcription in yeast but antagonizes PPAR signaling in mammalian cell," *Mol. Cell. Endocrinol.,* 120:31–39. 1996.

Miller, "Molecular biology of steroid hormone synthesis," *Endocr. Rev.,* 9:295–318. 1988.

Motojima, "Peroxisome proliferator-activated receptor (PPAR): Structure, mechanisms of activation and diverse functions," *Cell Structure and Function,* 18:267–277. 1993.

Nolan, Ludvik, Beerdsen, Joyce, Olefsky, "Improvement in glucose tolerance and insulin resistance in obese subjects treated with Troglitazone," *N. Engl. J. Med.*, 331:1188–1193, 1994.

Odell P F, Journal of Otolaryngology 25:7–13, 1996

Pierce W C, Figlin R A, Curr Opin Oncology 5:343–352, 1992

Rainey, Sawetawan, Shay, Michael, Mathis, Kutteh, Byrd, Carr, "Transformation of human granulosa cells with the E6 and E7 regions of human papillomavirus," *J. Clin. Endocrinol. Metabl.*, 78:705–710, 1994.

Shaaban M M, "The perimenopause and contratception" Maturitas 23:181–192, 1996

Urban, Bodenburg, Nagamani, Peirce, "Dexamethasone potentiates IGF-I actions in porcine granulosa cells," *Am. J. Physiol.*, 267:E115–E123, 1994.

Urban, Garmey, Shupnik, Veldhuis, "Insulin-like growth factor type I increases concentrations of messenger RNA encoding cytochrome P450 cholesterol side chain cleavage enzyme in primary cultures of porcine granulosa cells," *Endocrinology*, 127:2481–2488, 1990.

Veldhuis and Furlanette, "Trophic actions of human somatomedin C/insulin-like growth factor I on ovarian cells: in vitro studies with swine granulosa cells," *Endocrinology*, 116:1235–1242, 1985.

Vidal-Puig, Jimenez, Linan, Lowell, Hamann, Hu, Spiegelman, Flier, Moller, "Regulation of PPAR gamma gene expression by nutrition and obesity in rodents," *J. Clin. Invest.*, 97:2553–2561, 1996.

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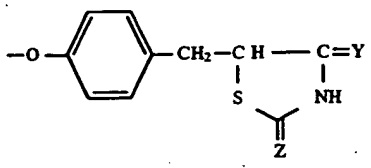


wherein R^1 and R^2 are the same or different and each represents a hydrogen atom or a C_1–C_5 alkyl group;

R^3 represents a hydrogen atom, a C_1–C_6 aliphatic acyl group, an alicyclic acyl group, an aromatic acyl group, a heterocyclic acyl group, an araliphatic acyl group, a (C_1–C_6 alkoxy)carbonyl group, or an aralkyloxycarbonyl group;

R^4 and R^5 are the same or different and each represents a hydrogen atom, a C_1–C_5 alkyl group or a C_1–C_5 alkoxy group, or R^4 and R^5 together represent a C_1–C_4 alkylenedioxy group;

n is 1, 2 or 3;

W represents the —CH_2—, >CO, or CH—OR^6 group (in which R^6 represents any one of the atoms or groups defined for R^3 and may be the same as or different from R^3); and

Y and Z are the same or different and each represents an oxygen atom or an imino (=NH) group;

and pharmaceutically acceptable salts thereof.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i i i) NUMBER OF SEQUENCES: 1

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

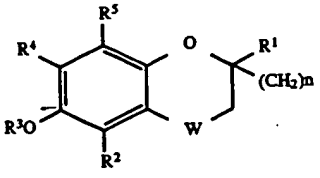
(i x) FEATURE:
(A) NAME/KEY: modified_base
(B) LOCATION: one-of(1, 2, 3, 10)
(D) OTHER INFORMATION: /note= "N = A, C, T or G"

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:1:

NNNAGGTCAN AGGTCA 16

What is claimed is:

1. A method of treating climacteric symptoms in a climacteric woman patient, comprising administering said patient a therapeutically effective amount of a compound of Formula I:



2. The method of 1 wherein the compound is in admixture with a pharmaceutically acceptable excipient, diluent, or carrier.

3. The method of claim 1 wherein Y and Z are oxygen.

4. The method of claim 1 wherein W is —CH_2—.

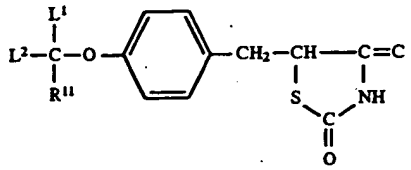
5. The method of claim 1 wherein n is 1.

6. The method of claim 1 wherein R^1, R^2, R^4, and R^5 are lower alkyl and R^3 is H.

7. The method of claim 1 wherein Z and Y are oxygen, n is 1, and W is —CH_2—.

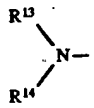
8. The method of claim 1 wherein the compound is (+)-5-[[4-[(3,4-dihydro-6-hydroxy-2,5,7,8-tetramethyl-2H-1-benzopyran-2-yl) methoxy]phenyl]methyl]-2,4-thiazolidinedione.

9. A method of treating climacteric symptoms in a climacteric woman patient, comprising administering said patient a therapeutically effective amount of a compound of Formula II:



Formula II

wherein R^{11} is substituted or unsubstituted alkyl, alkoxy, cycloalkyl, phenylalkyl, phenyl, aromatic acyl group, a 5- or 6-membered heterocyclic group including 1 or 2 heteroatoms selected from the group consisting of nitrogen, oxygen, and sulfur, or a group of the formula



wherein R^{13} and R^{14} are the same or different and each is lower alkyl or R^{13} and R^{14} are combined to each other either directly or as interrupted by a heteroatom selected from the group consisting of nitrogen, oxygen, and sulfur to form a 5- or 6- membered ring; and

wherein L^1 and L^2 are the same or different and each is hydrogen or lower alkyl or L^1 and L^2 are combined to form an alkylene group; or a pharmaceutically acceptable salt thereof.

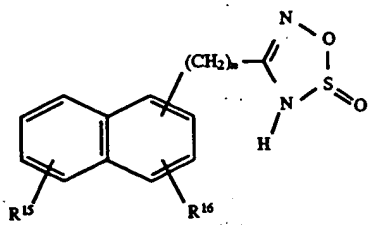
10. The method of claim 9 wherein the compound is in admixture with a pharmaceutically acceptable excipient, diluent, or carrier.

11. The method of claim 9 wherein the compound is troglitazone.

12. The method of claim 9 wherein the compound is pioglitazone.

13. The method of claim 9 wherein the compound is (±)-5-(14-{4-{2-Methyl-2(pyridylamino)ethoxy}methyl)2, 4-thiazolidinedione.

14. A method of treating a climacteric symptoms in a climacteric woman patient comprising administering said patient a therapeutically effective amount of a compound of Formula III:

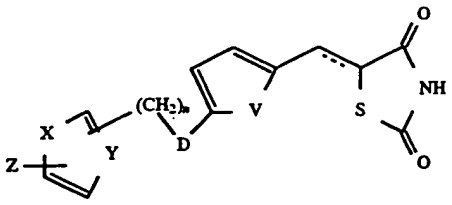


Formula III

wherein R^{15} and R^{16} are independently hydrogen, lower alkyl containing 1 to 6 carbon atoms,

alkoxy containing 1 to 6 carbon atoms, halogen, ethynyl, nitrile, methylthio, trifluoromethyl, vinyl, nitro, or halogen substituted benzyloxy; n is 0 to 4 and the pharmaceutically acceptable salts thereof.

15. A method f treating a climacteric symptoms in a climacteric woman patient comprising administering said patient a therapeutically effective amount of a compound of Formula IV:



Formula IV

wherein the dotted line represents a bond or no bond;

V is —CH=CH—, —N=CH—, —CH=N— or S;

D is CH_2, CHOH, CO, $C{=}NOR_{17}$ or CH=CH;

X is S, O, NR_{18}, —CH=N or —N=CH

Y is CH or N;

Z is hydrogen, $(C_1–C_7)$alkyl, $(C_3–C_7)$cycloalkyl, phenyl, naphthyl, pyridyl, furyl, thienyl, or phenyl mono- or disubstituted with the same or different groups which are $(C_1–C_3)$alkyl, trifluoromethyl, $(C_1–C_3)$alkoxy, fluoro, chloro, or bromo;

Z^1 is hydrogen or $(C_1–C_3)$alkyl;

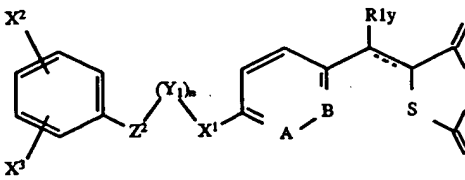
R^{17} and R^{18} are each independently hydrogen or methyl; and

n is 1, 2, or 3;

the pharmaceutically acceptable cationic salts thereof; and

the pharmaceutically acceptable acid addition salts thereof when the compound contains a basic nitrogen.

16. A method of treating a climacteric symptoms in a climacteric woman patient comprising administering said patient a therapeutically effective amount of a compound of Formula V:



Formula V

wherein the dotted line represents a bond or no bond;

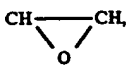
A and B are each independently CH or N, with the proviso that when A or B is N, the other is CH;

X^1 is S, SO, SO_2, CH_2, CHOH, or CO;

n is 0 or 1;

Y^1 is CHR_{20} or R^{21}, with the proviso that when n is 1 and Y^1 is NR_{21}, X^1 is SO_2 or CO;

Z^2 is CHR_{22}, CH_2CH_2, CH=CH,



OCH_2, SCH_2, $SOCH_2$ or SO_2CH_2;

R^{20}, R^{21}, and R^{22} are each independently hydrogen or methyl; and

X^2 and X^3 are each independently hydrogen, methyl, trifluoromethyl, phenyl, benzyl, hydroxy, methoxy, phenoxy, benzyloxy, bromo, chloro, or fluoro;

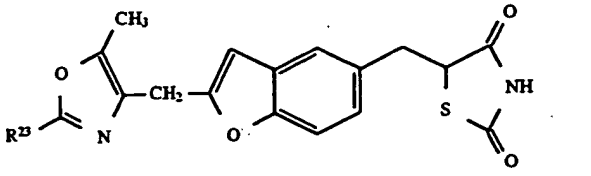
a pharmaceutically acceptable cationic salt thereof; or

a pharmaceutically acceptable acid addition salt thereof when A or B is N.

17. A method of treating a climacteric symptoms in a climacteric woman patient comprising administering said

patient a therapeutically effective amount of a compound of Formula VI:

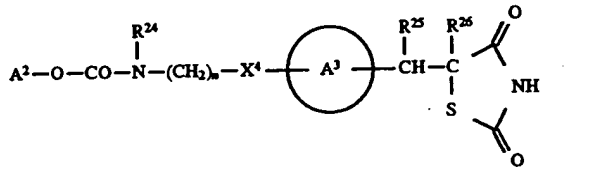
Formula VI



or a pharmaceutically acceptable salt thereof wherein R^{23} is alkyl of 1 to 6 carbon atoms, cycloalkyl of 3 to 7 carbon atoms, phenyl or mono- or disubstituted phenyl wherein said substituents are independently alkyl of 1 to 6 carbon atoms, alkoxy of 1 to 3 carbon atoms, halogen, or trifluoromethyl.

18. A method of treating a climacteric symptoms in a climacteric woman patient comprising administering said patient a therapeutically effective amount of a compound of Formula VII:

Formula VII



or a tautomeric form thereof and/or a pharmaceutically acceptable salt thereof, and/or a pharmaceutically acceptable solvate thereof, wherein:

A^2 represents an alkyl group, a substituted or unsubstituted aryl group, or an aralkyl group wherein the alkylene or the aryl moiety may be substituted or unsubstituted;

A^3 represents a benzene ring having in total up to 3 optional substituents;

R^{24} represents a hydrogen atom, an alkyl group, an acyl group, an aralkyl group wherein the alkyl or the aryl moiety may be substituted or unsubstituted, or a substituted or unsubstituted aryl group; or A^2 together with R^{24} represents substituted or unsubstituted C_{2-3} polymethylene group, optional substituents for the polymethylene group being selected from alkyl or aryl or adjacent substituents together with the methylene carbon atoms to which they are attached form a substituted or unsubstituted phenylene group;

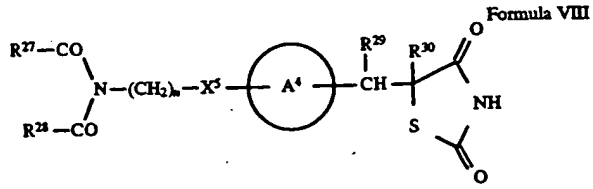
R^{25} and R^{26} each represent hydrogen, or R^{25} and R^{26} together represent a bond;

X^4 represents O or S; and

n represents an integer in the range from 2 to 6.

19. A method of treating a climacteric symptoms in a climacteric woman patient comprising administering said patient a therapeutically effective amount of a compound of Formula VIII in unit dosage form:

Formula VIII



or a tautomeric form thereof and/or a pharmaceutically acceptable salt thereof, and/or a pharmaceutically acceptable solvate thereof, wherein:

R^{27} and R^{28} each independently represent an alkyl group, a substituted or unsubstituted aryl group, or an aralkyl group being substituted or unsubstituted in the aryl or alkyl moiety; or R^{27} together with R^{28} represents a linking group, the linking group consisting of an optionally substituted methylene group or an O or S atom, optional substituents for the said methylene groups being selected from alkyl-, aryl, or aralkyl, or substituents of adjacent methylene groups together with the carbon atoms to which they are attached form a substituted or unsubstituted phenylene group;

R^{29} and R^{30} each represent hydrogen, or R^{29} and R^{30} together represent a bond;

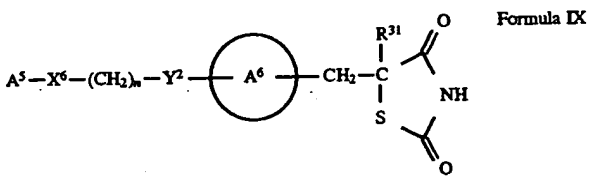
A^4 represents a benzene ring having in total up to 3 optional substituents;

X^5 represents O or S; and

n represents an integer in the range from 2 to 6.

20. A method of treating a climacteric symptoms in a climacteric woman patient comprising administering said patient a therapeutically effective amount of a compound of Formula IX:

Formula IX



or a tautomeric form thereof and/or a pharmaceutically acceptable salt thereof, and/or a pharmaceutically acceptable solvate thereof, wherein:

A^5 represents a substituted or unsubstituted aromatic heterocyclyl group;

A^6 represents a benzene ring having in total up to 5 substituents;

X^6 represents O, S, or NR_{32} wherein R^{32} represents a hydrogen atom, an alkyl group, an acyl group, an aralkyl group, wherein the aryl moiety may be substituted or unsubstituted, or a substituted or unsubstituted aryl group;

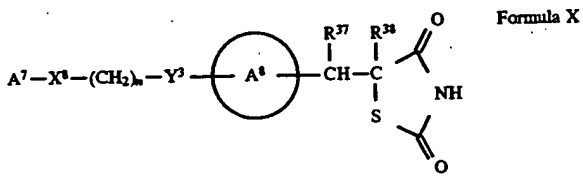
Y^2 represents O or S;

R^{31} represents an alkyl, aralkyl, or aryl group; and

n represents an integer in the range from 2 to 6.

21. A method of treating a climacteric symptoms in a climacteric woman patient comprising administering said patient a therapeutically effective amount of a compound of Formula X:

Formula X



or a tautomeric form thereof and/or a pharmaceutically acceptable salt thereof, and/or a pharmaceutically acceptable solvate thereof, wherein:

A^7 represents a substituted or unsubstituted aryl group;

A^8 represents a benzene ring having in total up to 5 substituents;

X^8 represents O, S, or NR_{39} wherein R^{39} represents a hydrogen atom, an alkyl group, an acyl group, an aralkyl group, wherein the aryl moiety may be substituted or unsubstituted, r a substituted or unsubstituted aryl group;

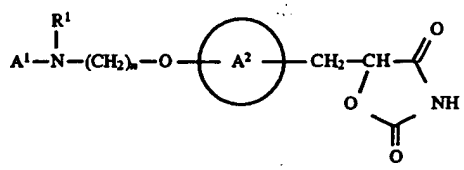
Y^3 represents O or S;

R^{37} represents hydrogen;

R^{38} represents hydrogen or an alkyl, aralkyl, or aryl group or R^{37} together with R^{38} represents a bond; and

n represents an integer in the range from 2 to 6.

22. A method of treating a climacteric symptoms in a climacteric woman patient comprising administering said patient a therapeutically effective amount of a compound of Formula XI:



Formula XI

or a tautomeric form thereof and/or a pharmaceutically acceptable salt thereof, and/or a pharmaceutically acceptable solvate thereof, wherein:

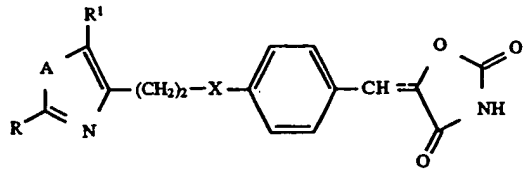
A^1 represents a substituted or unsubstituted heterocyclyl group;

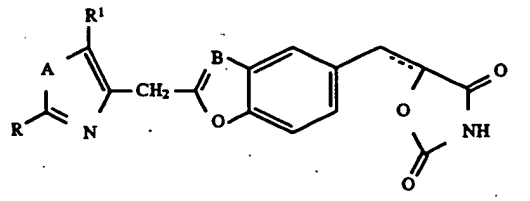
R^1 represents a hydrogen atom, an alkyl group, an acyl group, an aralkyl group, wherein the aryl moiety may be substituted or unsubstituted, or a substituted or unsubstituted aryl group;

A^2 represents a benzene ring having in total up to 5 substituents; and

n represents an integer in the range of from 2 to 6.

23. A method of treating a climacteric symptoms in a climacteric woman patient comprising administering said patient a therapeutically effective amount of a compound of Formula XII or XIII:





or a pharmaceutically acceptable salt thereof wherein the dotted line represents a bond or no bond;

R is cycloalkyl of three to seven carbon atoms, naphthyl, thienyl, furyl, phenyl or substituted phenyl wherein said substituent is alkyl of one to three carbon atoms, alkoxy of one to three carbon atoms, trifluoromethyl, chloro, fluoro or bis(trifluoromethyl);

R^1 is alkyl of one to three carbon atoms;

X is O or C=O;

A is O or S; and

B is N or CH.

24. A method of treating a climacteric symptoms in a climacteric woman patient comprising administering said patient a therapeutically effective amount of a compound selected from the group consisting of:

(+)-5-[[4-[(3,4-dihydro-6-hydroxy-2,5,7,8-tetramethyl-2H-1-benzopyran-2-yl) methoxy]phenyl]methyl]-2,4-thiazolidinedione: (troglitazone);

4-(2-naphthylmethyl)- 1,2,3,5-oxathiadiazole-2-oxide;

5-[4-[2-[N-(benzoxazol-2-yl)-N-methylamino]ethoxy] benzyl]-5-methylthiazolidine -2,4-dione;

5-[4-[2-[2,4-dioxo-5-phenylthiazolidin-3-yl)ethoxy] benzyl]thiazolidine-2,4-dione;

5-[4-[2-[N-methyl-N-(phenoxycarbonyl)amino]ethoxy] benzyl]thiazolidine-2,4-dione;

5-[4-(2-phenoxyethoxy)benzyl]thiazolidine-2,4-dione;

5-[4-[2-(4-chlorophenyl)ethylsulfonyl]benzyl] thiazolidine-2,4-dione;

5-[4-[3-(5-methyl-2-phenyloxazol-4-yl)propionyl] benzyl]thiazolidine-2,4-dione;

5-[4-[(1-methylcyclohexyl)methoxy]benzyl] thiadiazolidine-2,4-dione: (ciglitazone);

5-[[4-(3-hydroxy-1-methylcyclohexyl)methoxy]benzyl] thiadiazolidine-2,4-dione;

5-[4-[2-(5-methyl-2-phenyloxazol-4-yl)ethoxyl]benzyl] thiadizolidione-2,4-dione;

5-[4-[2-(5-ethylpyridin-2-yl)ethoxyl]benzyl] thiadiazolidine-2,4-dione: (pioglitazone);

5-[(2-benzyl-2,3-dihydrobenzopyran)-5-ylmethyl] thiadiazoline-2,4-dione: (englitazone);

5-[[2-(2-naphthylmethyl)benzoxazol]-5-ylmethyl] thiadiazoline-2,4-dione;

5-[4-[2-(3-phenylureido)ethoxyl]benzyl]thiadiazoline-2,4-dione;

5-[4-[2-[N-(benzoxazol-2-yl)-N-methylamino]ethoxy] benzyl]thiadiazoline-2,4-dione;

5-[4-[3-(5-methyl-2-phenyloxazol-4-yl)propionyl] benzyl]thiadiazoline-2,4-dione;

5-[2-(5-methyl-2-phenyloxazol-4-ylmethyl)benzofuran-5-ylmethyl]-oxazolidine -2,4-dione;

5-[4-[2-[N-methyl-N-(2-pyridyl)amino]ethoxy]benzyl] thiazolidine-2,4-dione (BRL49653); and

5-[4-[2-[N-(benzoxazol-2-yl)-N-methylamino]ethoxy] benzyl]-oxazolidine-2,4-dione.

* * * * *

Applicants

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[54] **FLOW CYTOMETRIC PHARMACOSENSITIVITY ASSAY AND METHOD OF CANCER TREATMENT**

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[21] Appl. No.: 559,812

[22] Filed: Nov. 17, 1995

[51] Int. Cl.[6] **A61K 38/19**; C12Q 1/06; G01N 33/52

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[58] Field of Search 424/85.1, 85.2, 424/85.4, 85.5, 85.6, 85.7; 435/29, 34, 39, 40.5, 4, 325, 347, 6; 436/813, 800, 172

[56] **References Cited**

U.S. PATENT DOCUMENTS

4,343,782	8/1982	Shapiro	424/3
4,665,020	5/1987	Saunders	435/7
5,069,662	12/1991	Bodden	604/4
5,229,265	7/1993	Tometsko	435/6

FOREIGN PATENT DOCUMENTS

WO 91/15595	10/1991	WIPO .
WO 95/20148	7/1995	WIPO .

OTHER PUBLICATIONS

Frankfurt et al. Abstract #1715, *Proceedings of the American Association For Cancer Research* (Mar. 1993), vol. 34, p. 288.

Holdaway, Karen M.; Finlay, Graeme J.; and Baguley, Bruce C., Relationship of Cell Cycle Parameters to in vitro and in vivo Chemosensitivity for a Series of Lewis Lung Carcinoma Lines (1992), *Eur. J. Cancer*, vol. 28A, No. 8/9, pp. 1427–1431.

Leone et al., Predictive Value of the Fluorescent Cytoprint Assay (FCA): A Retrospective Correlation Study of In Vitro Chemosensitivity and Individual Responses to Chemotherapy (1991), *Cancer Investigation*, 9(5), pp. 491–503.

Nrgaard et al. (Abstract), Synergistic and antagonistic effects of myeloid growth factors on in vitro cellular killing by cytotoxic drugs, *Leukemia Research* (1993), vol. 17, No. 8, pp. 689–694.

Afanasyev, V.N. et al., *Cytometry*. 14(6):603–9, 1993.

Boike, G.M. et al., *Proc. Annu. Meet. Am. Assoc. Cancer Res.* 30:A1857, 1989.

Bryson, G.J. et al., *Immunology & Cell Biology*. 72(1):35–41, 1994 Feb.

Choy, E.H. et al., *European Journal of Immunology*. 23(10):2676–81, 1993 Oct.

Dao, T. et al., *Cellular Immunology*. 155(2):304–11, 1994 May.

Darzynkiewicz, Z. et al., *Cytometry*. 13(8):795–808, 1992.

Elprana, D. et al., *Anticancer Research*. 12(6B) :2229–39, 1992.

Frankfurt, O. et al., *Proc. Annu. Meet. Am. Assoc. Cancer Res.* 33:A3261, 1992.

Frankfurt, O.S., *Proc. Annu. Meet. Am. Assoc. Cancer Res.* 29:A1942, 1988.

Frankfurt, O.S. et al., *Oncology Research*. 5(1):37–42, 1993.

Frankfurt, O. et al., *Proc. Annu. Meet. Am. Assoc. Cancer Res.* 34:A1715, 1993.

Garozzo, A. et al., *Journal of Chemotherapy*. 1(1):59–63, 1989.

Gheuens, E.E. et al., *Cytometry*. 12(7):636–44, 1991.

Gorczyca, W. et al., *Toxicology Letters*. 67(1–3):249–58, 1993 Apr.

Hedley, D.W., *Current Perspectives and Future Directions in Clinical Flow Cytometry*. Apr. 25–28, 1991, Baltimore, MD, A25.

Huschtscha, L.I. et al., *Experimental Cell Research*. 212 (1):161–5, 1994 May.

Lacombe, F. et al., *Proc. Annu. Meet. Am. Assoc. Cancer Res.* 33:A1201, 1992.

McCloskey, T.W. et al., *Clinical Immunology & Immunopathology*. 71(1):14–8, 1994 Apr.

Nicoletti, I. et al., *Journal of Immunological Methods*. 139(2):271–9, 1991 Jun 3.

(List continued on next page.)

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[57] **ABSTRACT**

A method of treating cancer by the use of a multidrug chemotherapeutic regimen determined by in vitro pharmacosensitivity tests. A cell suspension is prepared from a tumor specimen obtained from the patient. The viable tumor cell count within the cell suspension is calculated. The volume of the cell suspension is then adjusted to obtain a base cell concentration by diluting the cell suspension with patient medium in proportion with the viable tumor cell count. A sample of the cell suspension is retained as a negative control sample. Drug samples are then prepared, each drug sample containing a mixture of cell suspension, patient medium, and a drug selected from several drugs, wherein each drug sample contains a different drug which is added to the drug sample in an aliquot amount proportional to the base cell concentration. The drug samples and negative control sample are then incubated. After incubation, the drug samples and negative control sample are stained with a DNA intercalating dye. The cell viability in the drug samples and negative control sample is determined by use of a flow cytometer. The cell viability in the drug samples and negative control sample is compared to determine the pharmacosensitivity of the tumor. A multidrug treatment regimen is then administered to the patient, wherein the regimen includes the drugs shown to be most effective against the tumor in the pharmacosensitivity assay. The treatment has been shown to be especially useful in the simultaneous treatment of primary tumors and their metastases, especially when the chemotherapeutic regimen is administered loocoregionally by intra-arterial infusion methods.

14 Claims, No Drawings

OTHER PUBLICATIONS

Pilarski, L.M. et al., *Blood.* 83(3):724–36, 1994 Feb 1.

Riggs, C.E. Jr. et al. *American Society for Clinical Pharmacology & Therapeutics, Ninetieth Annual Meeting.* Mar. 8–10, 1989, Nashville, TN, p. 16.

Shimabukuro, T., *Hinyokika Kiyo–Acta Urologica Japonica.* 34(8):1349–55, 1988. 1988.

Sun, X.M. et al., *Analytical Biochemistry.* 204(2):351–6. 1992 Aug. 1.

Van de Loosdrecht, A.A. et al., *Experimental Hematology.* 21(13):1628–39, 1993 Dec.

Aabo, Kristian et al. "A Dominated and Resistant Subpopulation Causes Regrowth after Response to 1,3–Bis(2–chloroethyl)–1–nitrosourea Treatment of a Heterogeneous Small Cell Lung Cancer Xenograft in Nude Mice," *Cancer Research.* 54:3295–3299 (1994).

Arvelo, F. et al., "Response of a Multidrug–Resistant Human Small–Cell Lung Cancer Xenograft to Chemotherapy," *J. Cancer Res. Clin. Oncol.* 120:17–23 (1993).

Barry, Michael A. et al., "Etoposide–Induced Apoptosis in Human HL–60 Cells is Associated with Intracellular Acidification," *Cancer Research.* 53:2349–2357 (1993).

Bergamaschi, Gaetano et al., "Inhibitors of Tyrosine Phosphorylation Induce Apoptosis in Human Leukemic Cell Lines," *Leukemia.* 7:2012–2018 (1993).

Bradbury, Dawn et al., "Regulation of Bcl–2 Expression and Apoptosis in Acute Myeloblastic Leukaemia Cells by Granulocyte–Macrophage Colony–Stimulating Factor," *Leukemia.* vol. 8. pp. 786–791 (1994).

Bryson, Gregory J. et al., "A Flow Cytometric Study of Cell Death: Failure of Some Models to Correlate with Morphological Assessment," *Immunology and Cell Biology.* 72:35–41 (1994).

Campana, Dario et al., "Stroma–Supported Immunocytometric Assay (SIA): A Novel Method for Testing the Sensitivity of Acute Lymphoblastic Leukemia Cells to Cytotoxic Drugs," *Leukemia.* vol. 7:3 pp. 482–489 (1993).

Catchpoole, Daniel R. et al., "Etoposide–Induced Cytotoxicity in Two Human T–Cell Leukemic Lines: Delayed Loss of Membrane Permeability Rather than DNA Fragmentation as an Indicator of Programmed Cell Death," *Cancer Research.* 53:4287–4296 (1993).

Choy, Ernest H.S. et al., "Chimaeric Anti–CD4 Monoclonal Antibody Cross–Linked by Monocyte Fey Receptor Mediates Apoptosis of Human CD4 Lymphocytes," *Eur. J. Immunology.* 23:2676–2681 (1993).

Christ, M. et al., "Apoptosis Induced by Oxysterols in Murine Lymphoma Cells and in Normal Thymocytes," *Immunology.* 78:455–460 (1993).

Dao, T. et al., "Natural Human Interferon–α Augments Apoptosis in Activated T Cell Line," *Cellular Immunology.* 155:304–311.

Darzynkiewicz, Z. et al., "Features of Apoptotic Cells Measured by Flow Cytometry," *Cytometry.* 13:795–808 (1992).

Debatin, Klaus–Michael et al., "APO–1–Induced Apoptosis of Leukemia Cells from Patients with Adult T–Cell Leukemia," *Blood.* vol. 81 pp. 2972–2977 (1993).

DeVita, V.T. et al., *Cancer, Principles & Practice of Oncology.* 288–290.

Dole, Mukund et al., "Bcl–2 Inhibits Chemotherapy–Induced Apoptosis in Neuroblastoma," *Cancer Research.* 54:3253–3259 (1994).

Elprana, D. et al., "Chemosensitivity Testing of Xenografted Squamous Cell Carcinomas of the Head and Neck Region," *Anticancer Research.* 12:2229–2240 (1992).

Feldhoff, Pamela W. et al., "Altered Subcellular Distribution of Topoisomerase IIα in a Drug–Resistant Human Small Cell Lung Cancer Cell Line," *Cancer Research.* 54:756–762 (1994).

Fluckiger Anne–Catherine et al , "Interleukin 10 Induces Apoptotic Cell Death of B–Chronic Lymphocytic Leukemia Cells," *J. Exp. Med.* 179: 91–99 (1994).

Gong, Jianping et al., "Different Patterns of Apoptosis of HL–60 Cells Induced by Cycloheximide and Camptothecin," *Journal of Cellular Physiology.* 157:263–270 (1993).

Gorczyca Wojciech et al., "The Cell Cycle Related Differences in Susceptibility of HL–60 Cells to Apoptosis Induced by Various Antitumor Agents," *Cancer Research.* 53:3186–3192 (1993).

Gruber, Astrid et al., "Effect of Verapamil on Daunorubicin Accumulation in Human Leukemic Cells with Different Levels of MDRI Gene Expression," *Leukemia Research.* vol. 17 pp. 353–358 (1993).

Hamburger Anne W. et al., "Primary Bioassay of Human Tumor Stem Cells," *Science.* vol. 197, pp. 461–463.

Huschtscha, L. I. et al., "Identification of Apoptotic and Necrotic Human Leukemic Cells by Flow Cytometry," *Experimental Cell Research.* 212:161–165 (1994).

Hwang, Wei–Shou et al., "Prediction of Chemotherapy Response in Human Leukemia Using In Vitro Chemosensitivity Test," *Leukemia Research.* vol. 17:8 pp. 685–688 (1993).

Isobe, Hiroshi et al., "Doxorubicin Retention and Chemoresistance in Human Mesothelioma Cell Lines," *Int. J. Cancer.* 57:581–585 (1994).

Johnson, P.W.M. et al., "Isolated Follicular Lymphoma Cells are Resistant to Apoptosis and can be Grown In Vitro in the CD40/Stromal Cell System," *Blood.* vol. 82 pp.1848–1857 (1993).

Lacombe, Francis et al., "Detection of Cytarabine Resistance in Patients With Acute Myelogenous Leukemia Using Flow Cytometry," *Blood.* vol. 84 pp.716–723 (1994).

McCloskey, Thomas W. et al., "Use of a Flow Cytometric Assay to Quantitate Apoptosis in Human Lymphocytes," *Clinical Immunology and Immunopathology.* vol. 71 pp. 14–18 (1994).

Miyashita, T. et al., "Bcl–2 Oncoprotein Blocks Chemotherapy–Induced Apoptosis in a Human Leukemia Cell Line," *Blood.* vol. 81 pp. 151–157 (1993).

Nicoletti, I. et al., "A Rapid and Simple Method for Measuring Thymocyte Apoptosis by Propidium Iodide Staining and Flow Cytometry," *Journal of Immunological Methods.* 139:271–279 (1991).

Pantazis, Panayotis et al., "Regression of Human Breast Carcinoma Tumors in Immunodeficient Mice Treated with 9–Nitrocamptothecin: Differential Response of Nontumorigenic and Tumorigenic Human Breast Cells In Vitro," *Cancer Research.* 53:1577–1582 (1993).

Peters, G. J. et al., "Transformation of Mouse Fibroblasts with the Oncogenes H–ras or trk is Associated with Pronounced Changes in Drug Sensitivity and Metabolism," *Int. J. Cancer.* 54:450–455 (1993).

Raghu, Ganapathirama et al., "P–Glycoprotein and Alterations in the Glutathione/Glutathione–Peroxidase Cycle Underlie Doxorubicin Resistance in HL–60–R, a Subclone of the HL–60 Human Leukemia Cell Line," *Int. J. Cancer.* 53:804–911 (1993).

Schadendorf, Dirk et al., "Chemosensitivity Testing of Human Malignant Melanoma," *Cancer.* vol. 73:1 pp. 103–108 (1994).

Smit, E.F. et al., "In Vitro Response of Human Small–Cell Lung–Cancer Cell Lines to Chemotherapeutic Drugs; No Correlation With Clinical Data," *Int. J. Cancer.* 51:71–78 (1992).

Sparrow, Rosemary L. et al., "Common Expression of the Multidrug Resistance Marker P–Glycoprotein in B–Cell Chronic Lymphocytic Leukaemia and Correlation With In Vitro Drug Resistance," *Leukemia Research.* 17:941–947 (1993).

Sun, Xiao–Ming et al., "A Flow–Cytometric Method for the Separation and Quantitation of Normal and Apoptotic Thymocytes," *Analytical Biochemistry.* 204:351–356 (1992).

Takeda, Yuji et al., "Rapid Acceleration of Neutrophil Apoptosis Tumor Necrosis Factor–α," *International Immunology.* vol. 5 pp 691–694 (1993).

te Boekhorst, Peter A.W. et al., "Predominance of Functional Multidrug Resistance (MDR–1) Phenotype in CD34+ Acute Myeloid Leukemia Cells," *Blood.* vol. 82 pp. 3157–3162 (1993).

Toth, C.A. et al., "Type I Interferon Resistance in a Colorectal Cancer Cell Line is Associated With a More Aggressive Phenotype In Vivo," *Br. J. Cancer.* 65:365–368 (1992).

Uckun, Faith M. et al., "Autologous Bone Marrow Transplantation in High–Risk Remission B–Lineage Acute Lymphoblastic Leukemia Using a Cocktail of Three Monoclonal Antibodies Plus Complement and 4–Hydroperoxycyclophosphamide for Ex Vivo Bone Marrow Purging," *Blood.* vol. 79 pp. 1094–1104 (1992).

Van de Loosdrecht, A.A. et al., "Apoptosis in Tumor Necrosis Factor–α–Dependent, Monocyte–Mediated Leukemic Cell Death: A Functional, Morphologic, and Flow–Cytometric Analysis," *Experimental Hematology.* 21:1628–1639 (1993).

Vikhanskaya, Faina et al., "Introduction of Wild–Type p53 in a Human Ovarian Cancer Cell Line not Expressing Endogenous p53," *Nucleic Acids Research.* vol. 22 pp. 1012–1017 (1994).

Yamada, Toshiko et al., "The Human T–Cell Leukemia Virus Type I Tax Protein Induces Apoptosis Which is Blocked by the Bcl–2 Protein," *Journal of Virology.* pp. 3374–3379 (1994).

Zalcberg, John R. et al., "Cellular and Karyotypic Characterization of Two Doxorubicin Resistant Cell Lines Isolated From the Same Parental Human Leukemia Cell Line," *Int. J. Cancer.* 57:522–528 (1994).

Zhu, Y.M. et al., "Wild–Type p53 is Required for Apoptosis Induced by Growth Factor Deprivation in Factor–Dependent Leukaemic Cells," *Br. J. Cancer.* 69:468–472 (1994).

FLOW CYTOMETRIC PHARMACOSENSITIVITY ASSAY AND METHOD OF CANCER TREATMENT

FIELD OF THE INVENTION

The present invention relates generally to a method of in vivo treatment of cancer patients based on in vitro pharmacosensitivity testing, and more specifically to a method of cancer treatment with a chemotherapeutic regimen dictated by flow cytometric pharmacosensitivity tests.

DESCRIPTION OF THE PRIOR ART

PHARMACOSENSITIVITY ASSAYS

Cancer researchers have long been interested in discovering ways to predict the response of different individuals' tumors to different chemotherapeutic agents. However, tumors generally include cancer cells from numerous different tumor cell lines which live together in equilibrium. Different cell lines may be more resistant to chemotherapy than others. Thus, because certain individuals may have tumors with drug-resistant cell lines intermingled with "generic" tumor cell lines, medical personnel cannot simply assume that administration of a drug which is ordinarily effective against a certain type of tumor in most individuals will be effective in a particular individual suffering from that type of tumor. It has long been recognized that because of the heterogeneous nature of tumors and the likelihood of adaptive mutation due to the inherent instability of the tumor genome, a blanket or "off-the-shelf" phamaceutical agent for curing cancer, even a particular type of cancer, is unlikely. Several studies have expressly noted that blanket treatment of a group of patients with a specific anti-cancer drug or drugs often works very well for a subpopulation of the patients, but then either works minimally or not at all for the remainder of the patients because these patients suffer from drug-resistant tumors. Lacombe et al. (1994); Smit et al. (1992). As an example, doxorubicin is commonly administered for lung carcinoma due to its high effectiveness in certain cases, but for certain patients, treatment is virtually ineffective. In short, generic treatment methods frequently do not work with specific individuals because of varying tumor phenotypes.

In response to this problem, some researchers have proposed the use of in vitro pharmacosensitivity assays to substitute for the lack of in vivo dam for individual cancer cases: by testing an individual's tumor response to a chemotherapeutic agent in vitro, the individual's tumor response to that agent in vivo can supposedly be predicted. Most pharmacosensitivity assays currently in use are at least partially based on the pioneering soft agar tumor culture assay of Salmon et al. (1977). Other exemplary pharmacosensitivity assays are the MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) test and the dye exclusion assay test. Mossmann (1983); Smit et al., supra (1992); Hwang et al. (1993). The prior art contains several in vitro studies of tumor response according to these assays and correlates in vitro and in vivo results, e.g., Schadendorf et al. (1993).

Unfortunately, the prior art pharmacosensitivity assays suffer from several drawbacks which have prevented their widespread use.

First, as noted by the prior art, some assays are difficult, expensive, and time-consuming to perform. The last of these disadvantages is possibly the greatest hurdle to widespread use of pharmacosensitivity assays since medical personnel frequently cannot afford to expend extended amounts of time performing a pharmacosensitivity assay because of the threat of disease progression. Owing to this disadvantage, the remainder of this disclosure (and the invention disclosed herein) are addressed to a "short-term" assay, that is, a pharmacosensitivity assay that takes less than approximately one week to complete between the time of detection of the disease and the completion of the assay.

Second, as noted by many of the aforementioned studies, many of the prior art pharmacosensitivity assays are not applicable to all types of tumors, e.g., liquid tumors or very hard tumors. As a result, a specific prior art assay cannot be used for all patients, and different patients require different tests tailored to their specific types of tumors. This results in an increase in the overall cost of testing for all patients, and tends to have the effect of reserving assay-based treatment for the wealthy few who can afford such individualized care.

Third, the prior art pharmacosensitivity assays simply do not present an accurate reflection of in vivo drug application. Many of the assays suffer from low correlation between in vitro and in vivo results for particular (or all) tumor types and cell lines.

Due to the disadvantages listed above, the use of short-term pharmacosensitivity assays has been discouraged for use in cancer patient treatment, especially for the primary treatment of newly diagnosed patients; patients for whom it is believed that effective treatment exists; and for patients with a drug-sensitive tumor who fail the first trial of chemotherapy. See, e.g., DeVita et al. (1989). It is believed in the art that short-term pharmacosensitivity assays may provide a benefit insofar as they can deter patient exposure to the toxicity of drugs that are unlikely to be effective, but otherwise the assays cannot recommend a patient treatment regimen superior to one devised by an experienced medical practitioner who exercises sound judgment.

USE OF A MULTIDRUG CHEMOTHERAPEUTIC REGIMEN IN CANCER TREATMENT

It is known that a chemotherapeutic treatment regimen utilizing several drugs may be more effective than the best single drug. As summarized by Arvelo et al. (1993), this is due to several reasons. First, separate chemotherapeutic agents have different limiting toxicities and can therefore be combined in doses close to their maximum single-agent level. Second, one agent can fail to reach a disease confined to an organ, as if the tumor were in a pharmacological sanctuary, but other agents may be able to access the organ more efficiently. Third, a degree of potentiation exists between the agents in their efficacy against tumor cells to a greater extent than normal cells. Finally, different cellular mechanisms of resistance can be activated by different agents.

Those who treat cancer are still left with the problem of choosing the chemotherapeutic drugs to be used in a multidrug treatment regimen. There are literally hundreds of potential chemotherapeutic agents to combine with a wide variety of possible individual dosages, resulting in an infinite number of possible multidrug regimens. Medical personnel are generally inclined to apply one or more drugs shown by the prior art to be effective alone or in combination against the particular type of tumor to be treated. However, as noted above, there is no guarantee that "off-the-shelf" treatments will be effective against particular individuals' tumor cell lines. The use of a pharmacosensitivity assay

could help medical personnel to choose the most effective drugs for use in a multidrug treatment regimen, but the prior art pharmacosensitivity assays, which generally require considerable time and expense to screen the effectiveness of a single drug or a handful of drugs, are too inefficient to allow the screening of dozens of drugs. In view of the above considerations, those who suffer from cancer are in need of a method of cancer treatment which allows fast and easy determination and administration of an effective multidrug chemotherapeutic regimen which is particularly tailored to the needs of the individual, i.e., a method of cancer treatment which accounts for all of the unique specific cell lines within the individual's tumor.

APOPTOSIS

There are two major morphologically and biochemically distinct modes of death in nucleated eukaryotic cells: necrosis and apoptosis. Necrosis is essentially degenerative in nature and generally arises due to severe injury to cells. Apoptosis, on the other hand, is associated with the process of physiological cell death and appears to play a role opposite that of mitosis. Apoptosis is a mechanism by which a variety of cell types are deleted during embryonic development (morphogenesis) and hormone-induced organic involution. It also occurs in a variety of normal adult tissues, in particular those with a high cell turnover and during normal aging. Van de Loosdrecht et al. (1993).

Since the efficacy of several chemotherapeutic drugs correlates with their ability to induce apoptosis in tumor cells, the study of apoptosis is of interest to cancer research. Darzynkiewicz et al. (1992). Several researchers have studied the apoptosis-inducing effects of various chemotherapeutic drugs. For example, Christ et al. (1993) studied the effect of oxysterols on apoptosis of RDM4 murine lymphoma and mouse thymocytes in vitro and paralleled the findings of the study with in vivo application. Elprana et al. (1992) performed a similar study on the effects of cisplatin and bleomycin on squamous cell carcinomas obtained from the head and neck region. Lacombe et al., supra (1994) compared the results of in vitro application of Ara-C to acute myeloid leukemia with in vivo results and found good correlation. Campana et al. (1993) tested the in vitro sensitivity of lymphoblasts from acute lymphoblastic leukemia in response to five anti-leukemic drugs and compared the results to in vivo response.

Other researchers have similarly investigated the effects of certain biological response modifiers, e.g., interferons, on apoptosis. Dao et al. (1994); Thoth et al. (1992); Fluckiger et al. (1994).

During research, apoptotic cells may be detected in several ways. First, apoptotic cells illustrate internucleasomal cleavage of nuclear DNA into nucleosome-sized fragments of 200 base pair oligonucleosomal subunits. This registers as a ladder-like pattern on agarose gel slabs following electrophoretic separation. Additionally, Nicoletti et al. (1991) describes a method for detecting apoptotic cells by flow-cytometric analysis of isolated nuclei stained with propidium iodide (PI), an intercalating DNA stain, in hypotonic buffer. The highly-charged PI molecules cannot penetrate the membranes of living cells but are able to penetrate the intact membranes of dead, i.e., apoptotic, cells. When excited by laser light from a flow cytometer at 488 nm, the PI emits red fluorescence at 540 nm, allowing the flow cytometer to easily detect and count the apoptotic cells. The prior art discloses modification of the Nicoletti et al., supra (1991) method wherein other DNA stains are used as well, for example, acridine orange, Hoechst 33342, and ethidium bromide. Darzynkiewicz et al., supra (1992); Bryson, (1994); Huschtscha et al. (1994); Sun et al. (1992).

SUMMARY OF THE INVENTION

The present invention is directed to a method for treating cancer comprising the following steps. A cancer cell suspension is prepared from a specimen taken from a human cancer patient's tumor. The cell suspension is purified of non-cancer cell components, e.g., blood cells, by separating these components out by use of monoclonal antibodies and magnetic beads, or other separation means. Several drug samples, hereinafter referred to as "drug samples," are then prepared from the cell suspension and several putative cancer cell growth-inhibiting drugs, i.e., drugs which are believed to be likely candidates for chemotherapeutic treatment of the tumor. Each drug sample contains a mixture of at least one drug and the cell suspension. The drug samples are then incubated. After incubation, the tumor cell viability of each drug sample is determined to obtain a measure of the tumor cell pharmacosensitivity (the tumor cell response) to the drugs. A drug treatment regimen is then selected for the patient on the basis of the measured tumor cell viabilities for the drug samples. The drugs used in the treatment regimen are chosen from the drugs corresponding to the drug samples having the lowest tumor cell viability. The drug treatment regimen is administered to the patient in an amount which is effective to inhibit the growth of the cancer.

The present invention is additionally directed to a method for treating cancer comprising the following steps. A cell suspension is prepared from a cancer specimen obtained from a human cancer patient. A sample of the cell suspension is retained as a control sample. Drug samples are then prepared from the cell suspension and several putative cancer cell growth-inhibiting drugs, that is, drugs which are of interest as potential candidates for a multidrug chemotherapeutic regimen. Each drug sample contains a mixture of the cell suspension and at least one drug. The control sample and drug samples are then incubated. After incubation, the control samples and drug samples are stained with a DNA intercalating dye, and the cancer cell viability in the control sample and the drug samples is determined by use of a flow cytometer. The cancer cell viability of each drug sample is compared to the cancer cell viability of the control sample to determine the efficacy of each drug against the cancer cells. A drug treatment regimen is then prepared for the patient containing selected drugs chosen from the several drugs used in the drug samples. The selected drugs correspond to the drug samples having the lowest cancer cell viability in comparison to the control sample. The drug treatment regimen is administered to the patient in an amount which is effective to inhibit the growth of the cancer.

The present invention is further directed to a method of treating cancer in human cancer patients comprising the following steps. First, a cell suspension is prepared from a cancer specimen taken from a human cancer patient. The viable cancer cell count within the cell suspension is calculated. The volume of the cell suspension is then adjusted to obtain a base cell concentration by diluting the cell suspension with patient medium in proportion with the viable cancer cell count. A sample of the cell suspension is retained as a negative control sample. Drug samples are then prepared, each drug sample containing a mixture of cell suspension, patient medium, and a drug selected from several putative cancer cell growth-inhibiting drugs. Each drug sample contains a different drug which is added to the drug sample in an aliquot amount proportional to the base cell

concentration. The drug samples and negative control sample are then incubated. After incubation, the drug samples and negative control sample are stained with a DNA intercalating dye. The cancer cell viability in the drug samples and negative control sample is determined by use of a flow cytometer. The cancer cell viability in the drug samples and negative control sample is compared to determine the pharmacosensitivity of the cancer cells. A drug treatment regimen is formulated by selecting one or more drugs from the several cancer cell growth-inhibiting drugs. The selected drugs correspond to the drug samples having the highest cancer cell pharmacosensitivity. The drug treatment regimen is then administered to the patient in an amount which is effective to inhibit the growth of the cancer.

The invention provides an exceptionally rapid and versatile method of determining and administering an effective multidrug chemotherapeutic treatment regimen to a patient suffering from cancer. It allows the choice of an individualized and optimized chemotherapeutic regimen for a cancer patient in the space of approximately 48–96 hours after surgery or after a biopsy specimen of the tumor is obtained, thereby minimizing the opportunity for disease progression as the procedure is being carried out. Because the choice of the drugs used for the regimen is dictated by the patient's own tumor, the treatment is individualized. Such treatment is preferred over an "off-the-shelf" multidrug treatment because (1) it accounts for all tumor cell lines, and (2) it accounts for drug-resistant tumor cell lines, including the drugs effective against such cell lines and excluding those that are ineffective. It is believed that every different tumor from every different patient has a unique biological behavior, and that the patient will not receive the best possible treatment unless this is recognized. The benefits of the procedure are demonstrated by the fact that 78% of the patients treated by use of the procedure have achieved total or partial remission. Additionally, the mutidrug regimen dictated by the procedure has low toxicity insofar as ineffective chemotherapeutic agents are eliminated from the regimen.

It has been found that the procedure is also highly effective in treating metastases from primary tumors. Quite often, chemotherapeutic treatment is directed primarily toward the primary tumor and only secondarily to metastases. As a result, even where the primary tumor is defeated, the patient proceeds to perish from the metastastatic tumors. This is because treatment tailored for a particular (and often organotropic) primary tumor may be ill-suited for the treatment of distant metastases elsewhere. By developing a treatment regimen on the basis of the sensitivity of the particular cell lines rather than the type or location of a primary tumor, the procedure allows the development of an effective treatment regimen for both primary and metastatic tumors.

The procedure is well-suited for everyday performance in a laboratory. Because the procedure may be used for all types of tumors, and because it may be run for numerous different patients continuously or simultaneously, it is cost-effective for use with practically all cancer patients. The cost of the procedure can be further reduced if one or more of the steps of the procedure are automated.

Apart from the treatment of cancer, the procedure is expected to have application in the treatment of adjuvant therapy, wherein treatment is applied to an individual without evidence of disease due to a high likelihood of the presence of microscopic metastases. As an example, the procedure is expected to have value in the treatment of those who suffer from the Human Immunodeficiency Virus (HIV) where cancer is believed to be present.

The procedure is also useful for assessing the general efficacy of proposed new medications. Presently, proposed chemotherapeutic agents are tested in a broad range of concentrations against approximately 60 different tumor cell lines in a panel performed at the National Cancer Institute. The U.S. Food and Drug Administration (FDA) then uses small animals and rodents to test toxicity. The overall testing process takes a very long time, and as a result, the time lapse between discovery and actual use of a new medication takes an extremely long time. The procedure may be used to rapidly test proposed new chemotherapeutic agents against bone marrow cells to determine the toxicity of the agent, and against different cell lines to determine its efficacy. Because the basic procedure only takes an average of 72 hours to perform, the drug testing process can be greatly accelerated.

Further advantages, features, and objects of the invention will be apparent from the following detailed description of the invention.

DETAILED DESCRIPTION OF THE INVENTION

DEFINITIONS

The following definitions are intended to assist in providing a clear and consistent understanding of the scope and detail of certain terms used throughout this specification.

Biological Response Modifiers: Substances whose origin lies in the human (or animal) body, or which are man-made and mimic particular biological functions of such substances. Biological response modifiers have synergistic effect and produce increased tumor cell kill with a number of chemotherapeutic agents with little, if any, added side effects; have antitumor activity of their own; enhance populations of immune effector cells, such as natural killer cells and cytotoxic T cells; and lower levels of tumor growth factors such as insulin-like growth factor I. Perhaps the best-known biological response modifiers are interferons, a family of over 50 closely related glycoproteins with antiviral, immunoregulatory and antiproliferative functions. The immunoregulatory functions of interferons, such as the enhancement of natural killer lymphocyte activity, the increase in histocompatibility antigens, the activation of monocytes/macrophages, and B cell functions have proven to be of clinical importance, for example, in protecting bone marrow from the toxicity of chemotherapy. As an example, interferon alpha (IFNα) has been found to result in a considerable percentage of clinical remission alone or in combination with other drugs in diseases such as hairy-cell leukemia, chronic myelogenic leukemia, non-Hodgkin's lymphoma, multiple myeloma, and essential thrombocytosis.

The invention includes, but is not limited to, the following biological response modifiers, supplied by the following exemplary manufacturers:

- G-CSF, granulocyte-colony stimulating factor or filgrastim, by Amgen, Inc. (Thousand Oaks, Calif. 91320) under the trademark "NEUPOGEN";
- GM-CSF, granulocyte macrophage-colony stimulating factor or sargramostim, such as the recombinant human GM-CSF produced by Immunex Corp. (Seattle, Wash. 98101) under the trademark "LEUKINE";
- nHuIFNα (natural human leukocyte interferon alpha), by Virogen A. G. (Basel, Switzerland);
- nHuIFNαn-3 (natural human leukocyte interferon alpha n-3), by Purdue Frederick Co. under the trademark "ALFERON" (Norwalk, Conn. 06856);

nHuIFNβ (natural human interferon beta or natural human fibroblastic interferon), by Virogen Labs., Basel, Switzerland;

rIFNα-2a (recombinant interferon alpha-2a), by Roche Laboratories, a division of Hoffman-LaRoche Inc. (Nutley, N.J. 07110) under the trademark "ROFERON-A";

rIFNα-2b (recombinant interferon alpha-2b), by Schering Corp. (Kenilworth, N.J. 07033) under the trademark "INTRON-B";

rIFNβ-1b (recombinant interferon beta-1b or fiblaferon), by Berlex Labs. (Richmond, Calif. 94804) under the trademark "BETASERON";

rIFNτ-1b (recombinant interferon gamma-1b or polyferon), by Genentech, Inc. (San Francisco, Calif. 94080) under the trademark "ACTIMMUNE";

rIL-2 (interleukin 2 or aldesleukin) by Chiron Therapeutics (Emeryville, Calif. 94608); and

somatostatin, or analogues such as octreotide acetate, by Sandoz Pharmaceuticals, Inc. (East Hanover, N.J. 07936) under the trademark "SANDOSTATIN."

The invention further includes the following biological response modifiers which have been under clinical investigation:

nHuIFNπ-(natural human interferon pi, or antitumor specific interferon), the subject of copending U.S. patent application 07/179,529;

rTNF (recombinant tumor necrosis factor), by Chiron Therapeutics (Emeryville, Calif. 94608).

rIL-3 (interleukin 3), by Sandoz Pharmaceuticals, Inc. (East Hanover, N.J. 07936).

Drug: Throughout this specification, the term "drug" is understood to mean any substance used in the diagnosis, treatment, cure, and prevention of cancer. More specifically, the term "drug" will be used to apply to substances which are known or suspected to directly or indirectly prevent or inhibit the growth of cancer cells, either by directly attacking the cancer cells, by stimulating the body's immune system, or by other means. It is understood that "drug" also encompasses substances which are not believed to prevent or inhibit the growth of cancer cells by themselves, but which are known or suspected to do so when used in combination with one or more other drugs (e.g., mesna, discussed below). The term includes, but is not limited to, chemotherapeutic drugs recognized by the prior art, as well as the biological response modifiers listed above, alkylating agents, antibiotics, antimetabolites, and palliatives or drugs used to combat the side effects of chemotherapy (e . g., antiemetics, antidiarrhetics, hematological growth factors, etc.). As examples, a list of some of the drugs which can be used in the invention follows:

Amethopterin (e.g., "METHOTREXATE" by Lederle Labs.): an antifolate and one of the major antimetabolites used in cancer therapy. Amethopterin is commonly used for treating carcinoma of the breast, head, neck, lung, cervix, penis, prostate, testis, and bladder; acute lymphocytic leukemia; meningeal leukemia; non-Hodgkin's lymphoma; mycosis fungoides; osteosarcoma; and trophoblastic tumors.

Ara-C (Cytosine Arabinoside or Cytarabine, e.g., "CYTOSAR" by Upjohn Co., Kalamazoo, Mich. 49001): an antimetabolite commonly used to promote remission of acute non-lymphocytic (myelocytic) leukemia in adults and children. It has als been found useful in the treatment of acute lymphocytic leukemia, the blast phase of chronic myelocytic leukemia, and non-Hodgkin's lymphoma, but is inactive in most solid tumors.

BCNU (bis(chloroethyl)-nitrosurea or Carmustine, e.g., "BI CNU" by Bristol-Myers Oncology, a Bristol-Myers company, Evansville, Ind. 47721): an alkylating agent commonly used in palliative therapy as a single agent or in combination therapy with other chemotherapeutic drugs. It is also used for treating brain tumors, multiple myelomas, Hodgkin's Disease, and Non-Hodgkin's lymphomas.

Bleomycin (e.g., "BLENOXANE" by Bristol-Myers Oncology, a Bristol-Myers company, Evansville, Ind. 47721): an antitumor antibiotic commonly used in palliative treatment. It has been shown to be useful in the management of squamous cell carcinoma as well as testicular carcinomas and lymphomas.

Cis-platin (cis-platinum, e.g., "PLATINOL" by Bristol-Myers Oncology, a Bristol-Myers company, Evansville, Ind. 47721): a coordination complex of platinum (cisdiaminedichloroplatinum II) commonly used as a palliative treatment for testicular tumors, ovarian tumors and advanced bladder cancer. It has also been shown to promote regression in head and neck cancer as well as lung, cervical, and gastric cancers.

Cladribine (e.g., "LEUSTATIN" by Onho-Biotech, Raritan, N.J. 08869): a cytotoxic drug commonly used for treating hairy cell leukemia.

Cyclophosphamide (e.g., "CYTOXAN" by Bristol-Myers Oncology, a Bristol-Myers company, Evansville, Ind. 47721): an alkylating agent commonly used for treating malignant lymphomas, multiple myeloma, leukemias, neuroblastomas, adenocarcinomas of the ovary, retinoblastomas and breast carcinomas. Although effective alone in susceptible malignancies, it is more frequently used concurrently or sequentially with other antiplastic drugs.

Dactinomycin (Actinomycin-D, e.g., "COSMEGEN" by Merck Sharp & Dohme, West Pointe, N.Y. 19486): a cytotoxic crystalline antitumor antibiotic commonly used for treating Wilm's Tumor, and also in combination with other drugs for cancer of the testes and uterus, as well as melanomas and sarcomas.

Doxorubicin (e.g., "ADRIAMYCIN" by Adria Labs., Columbus, Ohio 43216): one of the most commonly used cytotoxic drugs, doxorubicin is an antitumor antibiotic commonly used for treating bladder, breast, head, neck, liver, lung, ovarian, prostatic, stomach, testicular and thyroid cancer, as well as Hodgkin's disease, leukemia, Wilm's tumor, lymphomas and sarcomas.

DTIC (e. g., "DACARBAZINE" by Miles Inc., West Haven, Conn. 06516): an alkylating agent commonly used in treatment of metastatic malignant melanoma and Hodgkin's disease.

Etoposide (VP-16, e.g., "VEPESID" by Bristol-Myers, Evansville, Ind. 47721): a cytotoxic epipodophyllotoxin (podophyllotoxin derivative from the mandrake plant) commonly used for treating carcinoma of the lung & testes.

Fludarabine phosphate (e.g., "FLUDARA" by Berlex Labs., Richmond, Calif. 94804): Fludarabine phosphate is a purine analog antimetabolic commonly used in the treatment of chronic lymphocytic leukemia (CLL). Other purine analogs such as 6-MP, 6-TG, azathiprine, allopurinol, acyclovir, gancylovir, deoxycoformycin, and arabinosyladienine (ara-A) have also been shown to have medical use and may also be suitable for use in the invention.

5-FU (5-fluorocytosine, e.g., "FLUOROURACIL" by Roche Labs., a division of Hoffman-LaRoche, Inc., Nutley, N.J. 07110): a cytotoxic fluoropyrimidine antimetabolic commonly used in the palliative management of carcinoma of the colon, rectum, breast, ovarian, cervix, bladder, stomach, liver and pancreas, 5-FU has synergistic interac-

tion with other antineoplastic agents, interferons, and irradiation and is thus commonly used in combination therapy.

Floxuridine (e.g., "FUDR" by Roche Labs., a division of Hoffman-LaRoche, Inc., Nutley, N.J. 07110): a cytotoxic drug commonly used in the palliative management of gastrointestinal adenocarcinoma metastatic to the liver. Also used for treating brain, breast, head and neck cancers with liver metastases.

Hydrea (Hydroxyurea, e.g., by E. R. Squibb & Sons, Princeton, N.J. 08543): a cytotoxic drug with demonstrated tumor response for melanoma, CML (chronic myelogenous leukemia) and recurrent metastatic carcinoma of the ovary.

Idamycin (Idarubicin, e.g., by Adria Labs., Columbus, Ohio 43216): a cytotoxic antitumor antibiotic commonly used in combination with other antileukemic drugs in treatment of acute myeloid leukemia in adults (most commonly Ara-C).

Ifosfamide (e.g., "IFEX" by Bristol-Myers Oncology, a Bristol-Myers company, Evansville, Ind. 47721): an alkylating agent commonly used in combination with other approved antineoplastic agents for third line chemotherapy of testicular cancer.

Levamisole (teramisole, e.g., "ERGAMISOLE" by Janssen Pharmaceuticals, Titusville, N.J. 08560): an immunomodulator/immunopotentiator; commonly used in combination with 5-FU after surgical resection in Dukes' stage C (tumor-node-metastasis stage III) colon cancer.

Mechlorethamine hydrochloride (nitrogen mustard, e.g., "MUSTARGEN" by Merck Sharp & Dohme, West Pointe, N.Y., 19486): a cytotoxic drug formerly used as a vesicant in chemical warfare, mechloretamine hydrochloride is an alkylating agent commonly used for treating lung carcinoma; chronic lymphocytic leukemia; chronic myelocytic leukemia; Hodgkin's lymphoma; non-Hodgkin's lymphoma; malignant effusions; and mycosis fungoides.

Medroxyprogesterone (e.g., "DEPO-PROVERA" by Upjohn Co., Kalamazoo, Mich. 49001): a hormone commonly used to control abnormal uterine bleeding due to hormonal imbalance, and in adjunctive therapy and palliative treatment of inoperable, recurrent, and metasiatic endometrial or renal carcinoma.

Megestrol Acetate (progestogen, e.g., "MEGACE" by Bristol-Myers Oncology, a Bristol-Myers company, Evansville, Ind. 47721): a hormone commonly used to treat metasiatic breast cancer.

Melphalan (e.g., "ALKERAN" by Burroughs Wellcome, Research Triangle Park, N.C. 27709): a derivative of mechlorethamine hydrochloride, melphalan is an alkylating agent commonly used for treating the palliative treatment of multiple myeloma and non-resectable epithelial carcinoma of the ovary.

Mesna (e.g., "MESNEX" by Bristol-Myers Oncology, a Bristol-Myers company, Evansville, Ind. 47721): commonly used as a prophylactic agent in reducing the incidence of ifosfamide-induced hemorrhagic cystitis.

Mito-C (Mitomycin-C, e.g., "MUTAMYCIN" by Bristol-Myers Oncology, a Bristol-Myers company, Evansville, Ind. 47721): an antitumor antibiotic that functions as a cytotoxic alkylating agent, mito-C is commonly used for treating gastric carcinoma and pancreatic carcinoma.

Octreotide acetate (an octapeptide analog of somatostatin, e.g., "SANDOSTATIN" by Sandoz Pharmaceuticals, East Hanover, N.J. 07936): a biological response modifier commonly used in metasiatic carcinoid tumors, vasoactive intestinal peptide tumors, and as a reducing growth hormone.

Paraplatin (e.g., "CARBOPLATIN" by Bristol-Myers Oncology, a Bristol-Myers company, Evansville, Ind. 47721): a platinum analog commonly used in palliative treatment of ovarian carcinoma as a single agent or in combination with other chemotherapy drugs.

Prednisone (e.g., by Schering Corp., Kenilworth, N.J. 07033): a hormone commonly used for treating nervous system disorders, GI disorders, edematous states, neoplastic diseases, hematological diseases, respiratory diseases, endocrine disorders, rheumatic disorders, collagenic diseases, dermalogical diseases, opthalmic diseases and allergic states, tuberculosis, and tricamonis.

Retinoic acid (e.g., isotretinoin or "ACCUTANE" by Roche Labs., a division of Hoffman-LaRoche, Inc., Nutley, N.J. 07110): commonly used for treating treatment of severe recalcitrant nodular acne that has been unresponsive to conventional treatments, i.e., antibiotics, etc. Retinoic acid is not a cytotoxic drug.

Streptozocin (e.g., "ZANOSAR" by Upjohn, Kalamazoo, Mich. 49001): a cytotoxic drug commonly used for treating pancreatic carcinoma.

Tamoxifen (tamoxifen citrate, e.g., "NOLVADEX" by ICI Pharma, Wilmington, Del. 19897): a cytotoxic drug commonly used for treating breast carcinoma in postmenopausal women with positive estrogen receptor assay.

Taxol (e.g., "PACLITAXEL" by Bristol-Myers Oncology, a Bristol-Myers company, Evansville, Ind. 47721): a cytotoxic drug taken from the bark of the pacific yew and commonly used for treating the treatment of metasiatic carcinoma of the ovary after failure of first-line or subsequent chemotherapy.

Thio-TEPA (triethylencthiophosphoramide, e.g., "PARENTERAL" by Lederle): an alkylating agent commonly used for treating carcinoma of the bladder, breast and ovaries; Hodgkin's lymphoma; non-Hodgkin's lymphoma; and malignant effusions.

VBL (Vinblastine, e.g., "VELSAR" by Quad Pharmaceuticals, Inc., Indianapolis, Ind. 46268): a cytotoxic vinca alkaloid from the periwinkle plant commonly used for treating breast carcinoma; testicular carcinoma; Hodgkin's lymphoma; non-Hodgkin's lymphoma; mycosis fungoides; and trophoblastic tumors.

VCR (Vincristine, e.g., "VINCASAR" by Quad Pharmaceuticals, Inc., Indianapolis, Ind. 46268): a cytotoxic vinca alkaloid related to vinblastine and vindesine (desacetyl/vinblastine amide). Commonly used for treating Ewing's sarcoma; acute lymphocytic leukemia; Hodgkin's lymphoma; non-Hodgkin's lymphoma; neuroblastoma; soft tissue carcinomas; and Wilm's tumor.

Flow Cytometer: In common flow cytometers, a cell suspension is hydrodynamically forced into a stream wherein cells pass by a focal point one by one. A laser is focused upon this focal point, and as a cell passes in front of the laser, the laser light is scattered in a variety of directions. The scattered light is collected and amplified by collection optics/detectors and converted into electrical impulses. The electrical impulses can then be decoded by computer and analyzed to convey information about the cells. The flow cytometer can count the cells in the suspension and collect information on different characteristics of the cells within the suspension by detecting dyes and other markers with which the cells have been treated. For example, cell subpopulations may be excluded from certain flow cytometric analyses by marking the subpopulations and having the flow cytometer gate (subtract) them from the analysis. Despite the foregoing description of the construction and operation of a flow cytometer, it is understood that the invention may use any type of cytometer that duplicates the essential functions of the flow cytometer described. The flow cytometer utilized

in the invention is the FACSCAN Immunocytometry Systems flow cytometer (Becton Dickinson, Mountain View, Calif., U.S.A.) running Consort 30 flow cytometry software (also by Becton Dickinson). Recently, the Becton Dickinson CELLQUEST software run on a MACINTOSH QUADRA 650 has been used as the preferred flow cytometry analysis software for the invention. Other suitable flow cytometers are the Coulter XL (Coulter Electronics, Hialeah, Fla., U.S.A.) or the Ortho Cytron (Ortho Diagnostic Systems, Raritan, N.J., U.S.A.).

Tumor: Throughout this specification, the term "tumor" is understood to mean cancer cells or a collection of cancer cells, whether in solid or liquid form. Examples of the tumors which have been treated or tested by use of the invention are: bladder cancer; breast cancer; colon carcinoma; non-small cell lung cancer; pancreatic cancer; liver cancer (metastases); prostatic carcinoma; acute myeloid leukemia; chronic myelogenous leukemia; chronic lymphocytic leukemia; hepatocellular carcinoma; glioblastoma; non-Hodgkin's lymphoma; melanoma; osteogenic sarcoma; ovarian carcinoma; pleomorphic adenocarcinoma; and Waldenstrom's macroglobulinemia. All have responded to treatment with the procedure. It is expected that other tumors will be treated with the procedure as they are encountered.

PHARMACOSENSITIVITY ASSAY

The pharmacosensitivity assay provides an accurate in vitro indication of in vivo tumor response within 48–96 hours from the time a solid or liquid tumor specimen is obtained from the patient. To summarize the assay procedure, a cell suspension is prepared from a patient's tumor specimen. The viable cell count of the suspension is calculated and standardized to a desired level. Multiple sample tubes are prepared, each containing cell suspension and a drug against which the pharmacosensitivity of the tumor is to be tested. Negative control tubes containing cell suspension and positive control tubes containing cell suspension and a cytotoxic agent are also prepared. The sample and control tubes are then incubated. After incubation, dyes such as propidum iodide are added to the sample and control tubes and all tubes are run through a flow cytometer. If desired, the flow cytometer can be compensated beforehand to factor out the presence of white cells in the cell suspension by staining the cleukouspension with leukocyte-binding substances such as fluorescein-labeled monoclonal antibodies, analyzing the stained cell suspension in the flow cytometer, and using the results to gate the white cells from further analyses. The flow cytometer may count the apoptotic cells (or alternatively, the non-apoptotic cells if the appropriate dye is used) and thereby obtain a measure of the cell kill, i.e., the pharmacosensitivity of the tumor cells.

(1) MATERIALS

The following materials are recommended for the performance of the pharmacosensitivity assay of the treatment method:

- Laminar flow hood
- RPMI medium (IX)
- Patient culture medium
- Tissue culture transport medium
- Select-a-Perle pipetter
- Select-a-Pette tips
- Eppendorf pipers
- Eppendorf Combitips (2.5 ml, 5.0 ml, 12.5 ml)
- Falcon 2072 plastic test tubes (with caps for FACS analysis)
- 15 ml capped tubes
- 100×15 mm sterile petri dishes
- 50 ml capped conical tubes
- Homogenizer
- Trypan blue stain (0.4%)
- Hemocytometer with cover slips
- Microscopes (with light)
- Hand tally counter
- Histopaque 1077
- Pipet suction aid
- Refrigerated centrifuge
- CO_2 incubator
- Drugs (lists provided elsewhere in specification)
- Vortex mixer
- Refrigerator (2°–10° C.)
- Scalpel
- Sharps container
- Propidium iodide (Sigma P4170) (Sigma, St. Louis, Mo., U.S.A.)
- Phosphate buffered saline, ph 7.2 (PBS)
- Flow cytometer (FACScan) (Becton Dickinson, Mountain View, Calif., U.S.A.)
- Clay-Adams Sero-fuge II (Clay-Adams, Parsippany, N.J., U.S.A.)
- CD45-FITC labeled MAb
- Anti-cytokeratin-FITC labeled MAb
- FACSlyse (diluted 1 in 10 in deionized water) (Becton Dickinson, Mountain View, Calif., U.S.A.)

All equipment is sterilized where appropriate.

(2) REAGENT PREPARATION

The procedure utilizes several reagents, the preparation of which is described below.

Preparation of 0.05 mg/ml Propidium Iodide Reagent (components available from Gibco, Grand Island, N.Y., U.S.A.)

2.5 milligrams (mg) propidium iodide is added to 50 milliliters (ml) phosphate buffered saline (PBS), pH 7.2, in a 50 ml tube. The tube is gently mixed by inverting the tube several times. The tube is labeled with the date of preparation and the expiration date and stored at 4° C. Since propidium iodide is light sensitive, the tube should be wrapped in foil before storage. The propidium iodide reagent is stable for approximately one month.

Preparation of Patient Culture Medium (IMDM/RPMI 1640 (1×), 10% FBS) (components available from Sigma, St. Louis, Mo., U.S.A.)

AMOUNT	COMPONENT
500.0 ml	RPMI Medium 1640 (1X), liquid with L-Glutamine
500.0 ml	Iscove's Modified Dulbecco's Medium (IMDM) (1X), liquid with L-Glutamine with 25 mM HEPES buffer with 3,024 mg/L sodium bicarbonate without alpha-thioglycerol without beta-mercaptoethanol
100.0 ml	Fetal Bovine Serum (FBS)
10.0 ml	L-Glutamine - 200 mM (100X), liquid
10.0 ml	Penicillin-Streptomycin, liquid 10,000 units/ml Penicillin 10,000 µg/ml Streptomycin
5.0 ml	NEAA Mixture 100x
5.0 ml	MEM Vitamin Solution 100x
5.0 ml	Insulin-Transferrin-Sodium Selenite Media Supplement

250.0 ml of RPMI (1×) and 250.0 ml of IMDM in sterile graduated cylinders are added under a sterile laminar flow hood to a 500.0 ml sterile filter system with a 0.22 mm

cellulose acetate membrane with a 60 mm prefilter. FBS is added with sterile disposable pipettes. All other ingredients listed are to be added immediately prior to use. Phenol red is used to perform a visual check of medium color for appropriate pH. The prepared medium is stable for 3 months when refrigerated at 2°–8° C.

Preparation of RPMI 1640 Medium (1×) (10% RPMI 1640 (1×) G,G fortified) (components available from Sigma, St. Louis, Mo., U.S.A.)

AMOUNT	COMPONENT
500.0 ml	RPMI Medium 1640 (1X), liquid with L-Glutamine
50.0 ml	Fetal Bovine Serum
5.0 ml	Penicillin-Streptomycin, liquid 10,000 units/ml Penicillin 10,000 µg/ml Streptomycin
5.0 ml	L-Glutamine - 200 mL (100x), liquid
5.0 ml	Insulin-Transferrin-Sodium Selenite Media Supplement

All components are combined and thoroughly mixed under a sterile laminar flow hood. Phenol red is used to perform a visual check of medium color for appropriate pH. The prepared medium is stable for 3 months when refrigerated at 2°–8° C.

Preparation of Tissue Culture Transport Medium (RPMI 1640 (1×) 15% FBS) (components available from Sigma, St. Louis, Mo., U.S.A.)

AMOUNT	COMPONENT
500.0 ml	RPMI Medium 1640 (1X), liquid
75.0 ml	Fetal Bovine Serum
5.0 ml	Penicillin-Streptomycin, liquid 10,000 units/ml Penicillin 10,000 µg/ml Streptomycin
5.0 ml	L-Glutamine - 200 mM (100x), liquid

All components are combined and thoroughly mixed under a sterile laminar flow hood. Phenol red is used to perform a visual check of medium color for appropriate pH. The medium is aliquotted into 50 ml conical tubes in 20.0 ml amounts. The prepared medium is stable for 3 months when refrigerated at 2°–8° C.

(3) PREPARATION OF THE CELL SUSPENSION

The pharmacosensitivity assay may use tumor samples from any type of tumor, including solid tumors obtained during surgery or biopsy, bone marrow aspirates and trephines, ascitic fluid, pleural fluid, and other types of tumor samples. After the tumor is obtained and placed in tissue culture transport medium, the tumor sample is converted to a cell suspension upon which the steps of the assay are performed. The sample is preferably large enough that it can yield an optimum of 15 ml of 1×10^6 viable tumor cells per ml of cell suspension, the preparation of which is described below.

For a solid tumor, the following steps are taken to prepare the cell suspension. The tumor specimen is cut into pea-size portions in a sterile petri dish using a sterile scalpel and forceps. The original tissue culture transport medium is retained for later use. The tumor portions are placed in a glass conical tube that has a matched loose-fitting homogenizer. 5.0 ml of tissue culture transport medium is added. The tumor portions are then gently homogenized and transferred to a sterile 50 ml conical tube. When all of the tumor portions have been homogenized, the mixture is stirred well and allowed to settle undisturbed for one minute to allow clumps of tissue to settle out. The supernatant is pipetted into a sterile tube, diluted with patient culture and centrifuged at 1600 RPM for 5 minutes. The supernatant is removed and the cells are resuspended in RPMI 1640 (1×) medium.

For liquid tumor, such as ascites, bone marrow, etc., monocyte separation may be performed by density gradient centrifugation on Ficoll-Hypaque medium (Pharmacia, Dublin, Ohio, U.S.A.). 20–25 ml of Histopaque 1077 is placed in the appropriate number of capped sterile 50 ml conical cubes by use of a sterile pipet. Using another sterile pipet, the liquid tumor is gently layered at a 45 degree angle onto the histopaque using a 1:1 ratio of histopaque to specimen. The histopaque is then centrifuged at 1600 RPM for 20 minutes in a refrigerated centrifuge at 4° C. (brake on 2). The supernatant is then removed and discarded, and the cell interface is removed and placed in RPMI 1640 (1×) medium in a pre-labeled tube. The cells should be diluted with the RPMI 1640 (1×) medium quickly because the histopaque is toxic to the cells if left on them for extended periods of time. The cells are then washed twice with RPMI 1640 (1×) medium and centrifuged at 1600 RPM for 5 minutes (brake on 2). The supernatant is removed and discarded, and the cell pellet is resuspended in 5.0 ml of RPMI 1640 (1×) medium.

Solid tumors are sometimes very hard and difficult to homogenize. If that is the case, it has been found that the procedure set out above for liquid tumors can be used to recover more viable cells and to rid the culture of debris and/or red blood cells.

(4) ANALYSIS OF VIABLE CELL CONCENTRATION

In this step of the procedure, the percentage of viable tumor cells is measured using viability tests known to the art, such as the Trypan Blue test or the MTT test. In the preferred method, 0.1 ml of well-mixed cell suspension, 0.2 ml of 0.4% Trypan Blue stain, and 0.7 ml of patient culture medium are combined in a 12×75 ml glass tube to obtain a 1:10 dilution of the cell suspension. The cell suspension is mixed well and charged into one chamber of a hemocytometer. A light microscope is used to count the both the viable tumor cells (those which have not absorbed the Trypan Blue) and the total number of tumor cells (stained and unstained) in the four corner 1 mm^2 squares.

The viable cell count is then determined using the following equation:

Viable cell count = [Average # of viable cells per square] ×

[dilution factor (here, 10)] ×

[hemocytometer multiplication factor]

The total cell count can be similarly calculated, and used in conjunction with the viable cell count to calculate the percent viability of the cell suspension according to the following formula:

% Viability=[viable cell count/total cell count]×100%

As noted above, other viability tests such as the MTT test may be used in place of the Trypan Blue exclusion test.

(5) ADJUSTMENT OF VIABLE CELL CONCENTRATION

A viable cell concentration of 1×10^6 viable tumor cells per ml of cell suspension is preferred for the use in the assay because the drug aliquots used in the procedure are predicated on a base viable tumor cell concentration of 1×10^6 viable tumor cells per ml of cell suspension. Where a concentration of greater than 1×10^6 viable tumor cells per ml is present, the cell suspension is diluted with patient culture

medium to yield this concentration. The dilution is performed according to the following formula:

Final cell suspension volume = [starting cell suspension volume] ×

[starting cell concentration] +

[desired concentration (here 1×10^6)]

Patient medium is added to reach the calculated final cell suspension volume. Alternatively, if the viable cell concentration is below 1×10^6 viable tumor cells per ml of cell suspension, the drug aliquots are diluted accordingly as described below at step (7). This drug aliquot dilution step may be performed at this stage instead, if desired.

(6) PREPARATION OF CONTROL SAMPLES

0.8 ml of patient culture medium is added to each of three sterile 75×12 mm Falcon 2072 plastic tubes (each having a cap for flow cytometric analysis). 0.2 ml of cell suspension is added to each of these tubes, which are to be used as negative control samples.

A positive control sample is also prepared. The preparation of this positive control sample will be outlined below.

(7) PREPARATION OF DRUG SAMPLES

One sterile 75×12 mm Falcon 2072 plastic tube (having a cap for flow cytometric analysis) is labeled for each drug to be used in the test. An additional tube is labeled as a positive control tube. The tubes are preset by adding 0.1 ml of the appropriate drug solution (at the aliquot amount set out below) and 0.7 ml of patient medium. These preset tubes may be stored at 4° C. for 7 days if capped with sterile caps.

(a) Drug Solution Aliquots for Use in the Drug Samples: following is a list of exemplary drugs used in the procedure and the preferred aliquot amount for their use.

Abrin (used in positive control sample): The preferred cytotoxic agent for use in the positive control is abrin (toxalbumin). Abrin solution is prepared by diluting abrin with RPMI 1640 (1×) medium to a working concentration of 0.5 mg/ml. As noted above, 0.1 ml of this abrin solution is added to the positive control tube.

Amethopterin: is generally supplied as a sterile lyophilized powder. The powder is reconstituted with sterile distilled water to obtain a 25.0 mg/ml concentration, and then dissolved in RPMI 1640 (1×) medium to yield a final working concentration of 0.25 mg/3.0 ml. If pre-prepared, the amethopterin solution may be stored at room temperature. As noted above, the amethopterin drug sample tube is prepared by adding 0.1 ml of amethopterin solution to a drug tube.

Ara-C: is generally supplied as a sterile lyophilized powder. The powder is reconstituted with sterile distilled water to obtain a 20.0 mg/ml concentration, and then dissolved in RPMI 1640 (1×) to obtain a final working concentration of 0.60 mg/ml. If pre-prepared, the Ara-C solution may be stored at room temperature. As noted above, the Ara-C drug sample tube is prepared by adding 0.1 ml of Ara-C solution to a drug tube.

BCNU: is generally supplied as a sterile lyophilized powder. The powder is reconstituted with sterile distilled water to obtain a 3.3 mg/ml concentration, and then dissolved in RPMI 1640 (1×) medium to yield a final working concentration of 0.003 mg/3.0 ml. If pre-prepared, the BCNU solution may be stored in the refrigerator. As noted above, the BCNU drug sample tube is prepared by adding 0.1 ml of BCNU soluti n to a drug tube.

Bleomycin: is generally supplied as a sterile lyophilized powder. The powder is reconstituted with sterile distilled water to obtain a 5.0 u/ml concentration, and then dissolved in RPMI 1640 (1×) medium to yield a final working concentration of 0.05 u/ml. If pre-prepared, the bleomycin solution may be stored in the refrigerator. As noted above, the bleomycin drug sample tube is prepared by adding 0.1 ml of bleomycin solution to a drug tube.

Cis-platin: is generally supplied as a sterile lyophilized powder. The powder is reconstituted with sterile distilled water to obtain a 1.0 mg/ml concentration, and then dissolved in RPMI 1640 (1×) medium to yield a final working concentration of 0.03 mg/3.0 ml. If pre-prepared, the cis-platin solution may be stored at room temperature. As noted above, the cis-platin drug sample tube is prepared by adding 0.1 ml of cis-platin solution to a drug tube.

Cladribine: is generally supplied in 1.0 mg/ml vials. The cladribine is dissolved in RPMI 1640 (1×) medium to yield a final working concentration of 0.01 mg/ml. If pre-prepared, the cladribine solution may be stored in the refrigerator. As noted above, the cladribine drug sample tube is prepared by adding 0.1 ml of cladribine solution to a drug tube.

Cyclophosphamide: is generally supplied as a sterile lyophilized powder. The powder is reconstituted with sterile distilled water to obtain a 20.0 mg/ml concentration, and then dissolved in RPMI 1640 (1×) medium to yield a final working concentration of 0.020 mg/3.0 ml. If pre-prepared, the cyclophosphamide solution may be stored in the refrigerator. As noted above, the cyclophosphamide drug sample tube is prepared by adding 0.1 ml of cyclophosphamide solution to a drug tube.

Dactinomycin: is generally supplied as a sterile lyophilized powder. The powder is reconstituted with sterile distilled water to obtain a 0.5 mg/ml concentration, and then solved in RPMI 1640 (1×) medium to yield a final working concentration of 0.015 mg/ml. If pre-prepared, the dactinomycin solution may be stored at room temperature. As noted above, the dactinomycin drug sample tube is prepared by adding 0.1 ml of dactinomycin solution to a drug tube.

Doxorubicin: is generally supplied as a sterile lyophilized powder. The powder is reconstituted with sterile distilled water to obtain a 2.0 mg/ml concentration, and then dissolved in RPMI 1640 (1×) to yield a final working concentration of 0.02 mg/3.0 ml. If pre-prepared, the doxorubicin solution should be stored in the refrigerator. As noted above, the doxorubicin drug sample tube is prepared by adding 0.1 ml of doxorubicin solution to a drug tube.

DTIC: is generally supplied as a sterile lyophilized powder. The powder is reconstituted with sterile distilled water to obtain a 10.0 mg/ml concentration, and then dissolved in RPMI 1640 (1×) medium to yield a final working concentration of 0.1 mg/3.0ml. If pre-prepared, the DTIC solution may be stored at room temperature. As noted above, the DTIC drug sample robe is prepared by adding 0.1 ml of DTIC solution to a drug tube.

Etoposide: is generally supplied in either liquid or capsule form. The etoposide is dissolved in RPMI 1640 (1×) medium to yield a final working concentration of 0.20 mg/3.0 ml. If pre-prepared, the etoposide solution may be stored at room temperature. As noted above, the etoposide drug sample tube is prepared by adding 0.1 ml of etoposide solution to a drug tube.

Fludarabine phosphate: is generally supplied as a sterile lyophilized powder. The powder is reconstituted with sterile distilled water to obtain a 25.0 mg/ml concentration, and then dissolved in RPMI 1640 (1×) medium to yield a final working concentration of 0.25 mg/3.0 ml. If pre-prepared, the fludarabine phosphate solution may be stored in the refrigerator. As noted above, the fludarabine phosphate drug sample tube is prepared by adding 0.1 ml of fludarabine phosphate solution to a drug tube.

5-FU: is generally supplied as an injectable solution with a concentration of 50.0 mg/ml. RPMI 1640 (1×) medium is added to obtain a working concentration of 0.50 mg/3.0 ml. If pre-prepared, the 5-FU solution may be stored at room temperature. As noted above, the 5-FU drug sample tube is prepared by adding 0.1 ml of 5-FU solution to a drug tube.

Floxuridine: is generally supplied as a powder. The powder is reconstituted with sterile distilled water to obtain a 100.0 mg/ml concentration, and then dissolved in RPMI 1640 (1×) medium to yield a final working concentration of 0.2 mg/3.0 ml. If pre-prepared, the floxuridine solution may be stored in the refrigerator. As noted above, the floxuridine drug sample tube is prepared by adding 0.1 ml of floxuridine solution to a drug tube.

G-CSF: If supplied in lyophilized form, the G-CSF should be reconstituted following the manufacturer's instructions. Stock solution concentration may be variable. The G-CSF should be diluted with RPMI (1×) medium to obtain a working concentration of 200.0 u/ml. Sterility should be maintained throughout the preparation process. Working solutions may be frozen in 1.5 ml aliquots in sterile nalgene cryovials. Frozen dilutions are stable up to five years at −20° C. Subsequent dilutions of frozen stock dilutions cannot exceed the expiration date of the frozen stock solution. Thawed aliquots are stable for three months at 2°–8° C.

GM-CSF: If supplied in lyophilized form, the GM-CSF should be reconstituted following the manufacturer's instructions. Stock solution concentration may be variable. The GM-CSF should be diluted with RPMI (1×) medium to obtain a working concentration of 62.5 u/ml. Sterility should be maintained throughout the preparation process. Working solutions may be frozen in 1.5 ml aliquots in sterile nalgene cryovials. Frozen dilutions are stable up to five years at −20° C. Subsequent dilutions of frozen stock dilutions cannot exceed the expiration date of the frozen stock solution. Thawed aliquots are stable for three months at 2°–8° C.

Hydrea: is generally supplied as 500 mg capsules. The capsules are reconstituted with sterile distilled water to obtain a 50 mg/ml concentration, and then dissolved in RPMI 1640 (1×) medium to yield a final working concentration of 0.50 mg/ml. If pre-prepared, the hydrea solution may be stored at room temperature. As noted above, the hydrea drug sample tube is prepared by adding 0.1 ml of hydrea solution to a drug tube.

Idamycin: is generally supplied as a sterile lyophilized powder. The powder is reconstituted with sterile distilled water to obtain a 1.0 mg/ml concentration, and then dissolved in RPMI 1640 (1×) medium to yield a final working concentration of 0.001 mg/3.0 ml. If pre-prepared, the idamycin solution may be stored at room temperature. As noted above, the idamycin drug sample tube is prepared by adding 0.1 ml of idamycin solution to a drug tube.

Ifosfamide: is generally supplied as a sterile lyophilized powder. The powder is reconstituted with sterile distilled water to obtain a 50.0 mg/ml concentration, and then dissolved in RPMI 1640 (1×) medium to yield a final working concentration of 1.5 mg/ml. If pre-prepared, the ifosfamide solution may be stored in the refrigerator. As noted above. the ifosfamide drug sample tube is prepared by adding 0.1 ml of ifosfamide solution to a drug tube.

Levamisole: is generally supplied in 10.0 mg tablets. The tablets are reconstituted with sterile distilled water to obtain a 5.0 mg/ml concentration, and then dissolved in RPMI 1640 (1×) medium to yield a final working concentration of 0.05 mg/3.0 ml. If pre-prepared, the levamisole solution may be stored at room temperature. As noted above, the levamisole drug sample tube is prepared by adding 0.1 ml of levamisole solution to a drug tube.

Mechlorethamine hydrochloride: is generally supplied as a sterile lyophilized powder. The powder is reconstituted with sterile distilled water to obtain a 1.0 mg/ml concentration, and then dissolved in RPMI 1640 (1×) medium to yield a final working concentration of 0.01 mg/3.0 ml. If pre-prepared, the mechlorethamine solution may be stored at room temperature. As noted above, the mechlorethamine drug sample tube is prepared by adding 0.1 ml of mechlorethamine solution to a drug tube.

Medroxyprogesterone: is generally supplied as a sterile lyophilized powder. The powder is reconstituted with sterile distilled water to obtain a 150.0 mg/ml concentration, and then dissolved in RPMI 1640 (1×) medium to yield a final working concentration of 1.50 mg/3.0 ml. If pre-prepared, the medroxyprogesterone solution may be stored at room temperature. As noted above, the medroxyprogesterone drug sample tube is prepared by adding 0.1 ml of medroxyprogesterone solution to a drug tube.

Megestrol acetate: is generally supplied in tablet form. The tablets are reconstituted with sterile distilled water to obtain a 4.0 mg/ml concentration, and then dissolved in RPMI 1640 (1×) medium to yield a final working concentration of 0.008 mg/3.0 ml. If pre-prepared, the megestrol acetate solution may be stored at room temperature. As noted above, the megestrol acetate drug sample tube is prepared by adding 0.1 ml of megestrol acetate solution to a drug tube.

Melphalan: is generally supplied in 2.0 mg tablets. The tablets are reconstituted with sterile distilled water to obtain a 0.2 mg/ml concentration, and then dissolved in RPMI 1640 (1×) medium to yield a final working concentration of 0.0002 mg/3.0 ml. If pre-prepared, the melphalan solution may be stored in the refrigerator. As noted above, the melphalan drug sample tube is prepared by adding 0.1 ml of melphalan solution to a drug tube.

Mesna: is generally supplied in prepared vials. The mesna is dissolved in RPMI 1640 (1×) medium to yield a final working concentration of 3.0 mg/3.0 ml. If pre-prepared, the mesna solution may be stored in the refrigerator. As noted above, the mesna drug sample tube is prepared by adding 0.1 ml of mesna solution to a drug tube.

Mito-C: is generally supplied in 0.5 mg/ml vials. The mito-C is dissolved in RPMI 1640 (1×) medium to yield a final working concentration of 0.005 mg/ml. If pre-prepared, the mito-C solution may be stored at

room temperature. As noted above, the mito-C drug sample robe is prepared by adding 0.1 ml of mito-C solution to a drug tube.

nHuIFNα: If supplied in lyophilized form, the nHuIFNα should be reconstituted following the manufacturer's instructions. Stock solution concentration may be variable. The nHuIFNα should be diluted with RPMI (1×) medium to obtain a working concentration of 1.0×10^3 u/mL. Sterility should be maintained throughout the preparation process. Working solutions may be frozen in 1.5 ml aliquots in sterile nalgene cryovials. Frozen dilutions are stable up to five years at −20° C. Subsequent dilutions of frozen stock dilutions cannot exceed the expiration date of the frozen stock solution. Thawed aliquots are stable for three months at 2°–8° C.

nHuIFNβ: If supplied in lyophilized form, the nHuIFNβ should be reconstituted following the manufacturer's instructions. Stock solution concentration may be variable. The nHuIFNβ should be diluted with RPMI (1×) medium to obtain a working concentration of 1.0×10^3 u/mL. Sterility should be maintained throughout the preparation process. Working solutions may be frozen in 1.5 ml aliquots in sterile nalgene cryovials. Frozen dilutions are stable up to five years at −20° C. Subsequent dilutions of frozen stock dilutions cannot exceed the expiration date of the frozen stock solution. Thawed aliquots are stable for three months at 2°–8° C.

nHuIFNπ: If supplied in lyophilized form, the nHuIFNπ should be reconstituted following the manufacturer's instructions. Stock solution concentration may be variable. The nHuIFNπ should be diluted with RPMI (1×) medium to obtain a working concentration of 5.0×10^2 u/mL. Sterility should be maintained throughout the preparation process. Working solutions may be frozen in 1.5 ml aliquots in sterile nalgene cryovials. Frozen dilutions are stable up to five years at −20° C. Subsequent dilutions of frozen stock dilutions cannot exceed the expiration date of the frozen stock solution. Thawed aliquots are stable for three months at 2°–8° C.

Octreotide acetate: is prepared similarly to somatostatin, discussed below.

Paraplatin: is generally supplied as a sterile lyophilized powder. The powder is reconstituted with sterile distilled water to obtain a final concentration of 5.0 mg/ml, and then dissolved in RPMI 1640 (1×) medium to yield a final working concentration of 0.05 mg/mL. If pre-prepared, the paraplatin solution may be stored at room temperature. As noted above, the paraplatin drug sample tube is prepared by adding 0.1 ml of paraplatin solution to a drug tube.

Prednisone: is generally supplied in liquid form. The prednisone is dissolved in RPMI 1640 (1×) medium to yield a final working concentration of 0.6 mg/mL. If pre-prepared, the prednisone solution may be stored at room temperature. As noted above, the prednisone drug sample tube is prepared by adding 0.1 ml of prednisone solution to a drug tube.

Retinoic acid: is generally available in tablet form. The tablets are dissolved in RPMI 1640 (1×) medium to obtain a final working concentrati n of 0.04 mg/3.0 ml. If pre-prepared, the retinoic acid solution may be stored at room temperature. As noted above, the retinoic acid drug sample tube is prepared by adding 0.1 ml of retinoic acid solution to a drug tube.

rIFNα-2a: If supplied in lyophilized form, the rIFNα-2a should be reconstituted following the manufacturer's instructions. Stock solution concentration may be variable. The rIFNα-2a should be diluted with RPMI (1×) medium to obtain a working concentration of 30,000 u/ml. Sterility should be maintained throughout the preparation process. Working solutions may be prepared as needed in 17×100 sterile capped tubes. Frozen dilutions are stable up to five years at −20° C. Subsequent dilutions of frozen stock dilutions cannot exceed the expiration date of the frozen stock solution. Thawed aliquots are stable for three months at 2°–8° C.

rIFNα-2b: If supplied in lyophilized form, the rIFNα-2b should be reconstituted following the manufacturer's instructions. Stock solution concentration may be variable. The rIFNα-2b should be diluted with RPMI (1×) medium to obtain a working concentration of 1,000 neutralizing units/ml. Sterility should be maintained throughout the preparation process. Working solutions may be frozen in 3.5 ml aliquots in 12×75 sterile capped tubes. Frozen dilutions are stable up to five years at −20° C. Subsequent dilutions of frozen stock dilutions cannot exceed the expiration date of the frozen stock solution. Thawed aliquots are stable for three months at 2°–8° C.

rIFNβ-1b: If supplied in lyophilized form, the rIFNβ-1b should be reconstituted following the manufacturer's instructions. Stock solution concentration may be variable. The rIFNβ-1b should be diluted with RPMI (1×) medium to obtain a working concentration of 1.0×10^3 u/ml. Sterility should be maintained throughout the preparation process. Working solutions may be frozen in 1.5 ml aliquots in sterile nalgene cryovials. Frozen dilutions are stable up to five years at −20° C. Subsequent dilutions of frozen stock dilutions cannot exceed the expiration date of the frozen stock solution. Thawed aliquots are stable for three months at 2°–8° C.

rIFNτ-1b: If supplied in lyophilized form, the rIFNτ-1b should be reconstituted following the manufacturer's instructions. Stock solution concentration may be variable. The rIFNτ-1b should be diluted with RPMI (1×) medium to obtain a working concentration of 0.01 μg/ml. Sterility should be maintained throughout the preparation process. Working solutions may be frozen in 1.5 ml aliquots in sterile nalgene cryovials. Frozen dilutions are stable up to five years at −20° C. Subsequent dilutions of frozen stock dilutions cannot exceed the expiration date of the frozen stock solution. Thawed aliquots are stable for three months at 2°–8° C.

rIL-2: If supplied in lyophilized form, the rIL-2 should be reconstituted following the manufacturer's instructions. Stock solution concentration may be variable. The rIL-2 should be diluted with RPMI (1×) medium to obtain a working concentration of 10.0 u/ml. Sterility should be maintained throughout the preparation process. Working solutions may be frozen in 10 ml aliquots in 17×100 sterile capped tubes. Frozen dilutions are stable up to five years at −20° C. Subsequent dilutions of frozen stock dilutions cannot exceed the expiration date of the frozen stock solution. Thawed aliquots are stable for three months at 2°–8° C.

rTNF: If supplied in lyophilized form, the rTNF should be reconsituted following the manufacturer's instructions. Stock solution concentration may be variable. The rTNF should be diluted with RPMI (1×) medium to obtain a working concentration of 1.2×10^2 u/ml. Sterility should be maintained throughout the preparation process. Working solutions may be frozen in 1.5 ml

aliquots in sterile nalgene cryovials. Frozen dilutions are stable up to five years at –20° C. Subsequent dilutions of frozen stock dilutions cannot exceed the expiration date of the frozen stock solution. Thawed aliquots are stable for three months at 2°–8° C.

Somatostatin: If supplied in lyophilized powder form, the Somatostatin should be reconstituted following the manufacturer's instructions. Stock solution concentration may be variable. The somatostatin should be diluted with RPMI (1×) medium to obtain a working concentration of 50.0/μg/ml. Sterility should be maintained throughout the preparation process. Working solutions may be frozen in 1.5 ml aliquots in sterile nalgene cryovials. Frozen dilutions are stable up to five years at –20° C. Subsequent dilutions of frozen stock dilutions cannot exceed the expiration date of the frozen stock solution. Thawed aliquots are stable for three months at 2°–8° C.

If supplied in liquid form, somatostatin is provided in 50 ug/cc vials with diluent. The somatostatin is dissolved in RPMI 1640 (1×) medium to yield a final working concentration of 5.0 ug/3.0 ml. If pre-prepared, the somatostatin solution may be stored in the refrigerator. As noted above, the somatostatin drug sample tube is prepared by adding 0.1 ml of somatostatin solution to a drug tube.

Streptozocin: is generally supplied as a sterile lyophilized powder. The powder is reconstituted with sterile distilled water to obtain a 100.0 mg/ml concentration, and then dissolved in RPMI 1640 (1×) medium to yield a final working concentration of 1.0 mg/3.0 ml. If pre-prepared, the streptozocin solution may be stored in the refrigerator. As noted above, the streptozocin drug sample tube is prepared by adding 0.1 ml of streptozocin solution to a drug tube.

Tamoxifen: is generally supplied in 10.0 mg tablets. The tablets are reconstituted with sterile distilled water to obtain a 1.0 mg/ml concentration, and then dissolved in RPMI 1640 (1×) medium to yield a final working concentration of 0.003 mg/3.0 ml. If pre-prepared, the tamoxifen solution may be stored at room temperature. As noted above, the tamoxifen drug sample tube is prepared by adding 0.1 ml of tamoxifen solution to a drug tube.

Taxol: is generally supplied as a liquid. The taxol is dissolved in RPMI 1640 (1×) medium to yield a final working concentration of 0.06 mg/3.0 ml. If pre-prepared, the taxol solution may be stored in the refrigerator. As noted above, the taxol drug sample tube is prepared by adding 0.1 ml of taxol solution to a drug tube.

Thio-TEPA: is generally supplied as a sterile lyophilized powder. The powder is reconstituted with sterile distilled water to obtain a 2.5 mg/ml concentration, and then dissolved in RPMI 1640 (1×) medium to yield a final working concentration of 0.025 mg/ml. If pre-prepared, the thio-TEPA solution may be stored in the refrigerator. As noted above, the thio-TEPA drug sample tube is prepared by adding 0.1 ml of thio-TEPA solution to a drug tube.

VBL: is generally supplied as a sterile lyophilized powder. The powder is reconstituted with sterile distilled water to obtain a 1.0 mg/ml concentration, and then dissolved in RPMI 1640 (1×) medium to yield a final working concentration of 0.03 mg/ml. If pre-prepared, the VBL solution may be stored in the refrigerator. As noted above, the VBL drug sample tube is prepared by adding 0.1 ml of VBL solution to a drug tube.

VCR: is generally supplied in liquid form. The VCR is dissolved in RPMI 1640 (1×) medium to yield a final working concentration of 0.01 mg/ml. If pre-prepared, the VCR solution may be stored in the refrigerator. As noted above, the VCR drug sample tube is prepared by adding 0.1 ml of VCR solution to a drug tube.

Additional information regarding standard aliquots and dosages for any of the aforementioned drugs may found in the most current edition of the Physician's Desk Reference. It is expected that aliquots can be prepared for other chemotherapeutic drugs as well, examples being acivin, aclacinomycin A, alanine mustard, altretamine, aminoglutethimide, aminopterin, amsacrine (mAMSA), various anabolic steroids, anthrapyrazoles, L-asparaginase, 5-axacytidine, bacillus calmette-guerin, bisantrene, buserelin, busulfan, butyryloxyethylglyoxal ditiosemicarbazone, camptothecin, carbamate ester, carzinophyllin, CCNU, chlorambucil, chlorethyl-methyl-cycloexyl-nitrosourea, chlorethylcyclohexylnitrosourea, chlororderoxyadenosine, chlorotrianisene, chlorozotocin, chromic phosphate P32, chromomycin-A, cobalt proporphyrin complex, corticotropin, cyproterone acetate, daunomycin, daunorubicin, dehydroemitine, 4-demethoxydaunorubicin, demothydoxorubicin, deoxydoxorubicin, dexamethosone, dibromodulcitol, dichloromethotrexate, diethylstilbestrol, DL-serine bis(2-chloropropyl), doxifluridine, elliptinium acetate, 4'-epidoxorubicin, epirubicin, epoetin alpha, erythropoeitin, esorubicin, estradiol, estradiol valerate, estramustine, estrogens, estrone, ethynil estradiol, etidronate, fluoxymesterone, flutamide, folic acid, fotemustine, ftorafur, 4-FU, galactitol, gallium nitrate, goserelin, hexamethylmelamine (HMM), hydrocortisone, 4-hydroperoxycyclophosphamide, hydroxyprogesterone, ICRf 159, immune globulin IGIV, L-asparaginase, leucovorin calcium, leuprolide, levadopa, levothyroxine, lindane, liothyronine, liotrix, lomustine, masoprocol, maytansine, menogaril, 6-mercaptopurine, methosalen, methylesterone, methyl-lomustine, mithracin, mithramycin, mitotane, mitoxantrone, 6 MP, nandrolone, navelbine, neocarzinostatin, nitrofururazone, ondansetrone HCl, pamidronate disodium, pentamethylmelamine (PMM), pentostatin, peptiochemio, plicamycin, prednimustine, probroman, procarbazine, profiromycin, razoxane, retinoids (retinol and retinol analogs), rubidazone, semustine, sodium iodine I-131, sodium phosphate P32, SPG 827 (podophyllin derivative), spirogermanium, streptonigrin, 6-thioguanine, tenoposide, testolactone, testosterone, 3-TGDR, thioguanine, thyroglobulin, thyrotropin, trilostane, uracil mustard, verapamil, vindesine, vinzelidine, and zinostatin. Again, standard aliquots and dosages may be prepared as stated in the Physician's Desk Reference. Of course, other chemotherapeutic agents may be used as they become available.

Safety precautions should be followed at all times when handling the drugs. Most cytotoxic drugs are deemed to be carcinogenic and are severe irritants to exposed skin. Extreme care should be taken when handling these substances and personal protective clothing should be worn at all times. Special care must be taken when handling abrin, and all material coming into contact with abrin must be autoclaved after each use.

(b) Adjustment of Drug Concentration

If the viable cell concentration of the cell suspension is less than 1×10^6 cells/ml, it is necessary to reduce the drug concentration in each drug sample tube by the addition of more patient culture medium. Patient culture medium is

added to each tube in a logarithmic relationship to the viable cell concentration, with 1.0 ml of patient culture medium being added to each tube for each order of magnitude less than 1×10^6 cells/ml. The following table illustrates this relation:

Viable Cell Concentration (cells/ml)	Volume of Patient Culture Medium Added (ml)
1.0×10^6	0
7.5×10^5	0.25
5.0×10^5	0.5
2.5×10^5	0.75
1.0×10^5	1.0

Acceptable results may be obtained if the viable cell concentration is rounded to the closest amount listed in the table and the corresponding volume of patient culture medium is added. However, cell counts of less than 1×10^5/ml are not preferred.

(c) Completion of the Drug Samples

To complete the drug samples and the positive control sample, 0.2 ml of cell suspension is added to each tube.

(8) INCUBATION

The drug samples and positive and negative control samples are then mixed and incubated. An optimum incubation time and environment has been found to be 72 hours at 37° C. and 5% CO_2. Greater incubation times do not appear to yield significantly different results.

(9) COMPENSATION FOR WHITE CELL CONTENT OF THE SAMPLES

Because white cells present in the samples can skew the results of the flow cytometry readings, it is recommended that the flow cytometer be compensated for the white cell content of the tumor sample using the following steps. These steps need not be followed for bone marrow specimens because in that case, the white cells are the target cells to be measured.

0.2 ml of cell suspension is added to a 75×12 mm Falcon tube with 10 microliters of FITC (fluorescein isothiocyanate) labeled CD45 monoclonal antibody (MAb). The tube is incubated at room temperature for 15 minutes. 2.0 ml of FACSlyse solution (Becton Dickinson, Mountain View, Calif., U.S.A.), diluted 1:10 in deionized water, is added to the tube. The tube is again incubated at room temperature for 10 minutes. The tube is then centrifuged at high speed and the supernatant is decanted. The tube is washed once by adding 2.0 ml of PBS to the tube and centrifuging at high speed. The supernatant is decanted and 0.5 ml PBS is added.

A flow cytometer is then used to detect the FITC-marked white cells. The tube is placed on the flow cytometer and analyzed. A histogram of the FITC fluorescence versus the number of cells is created, wherein the CD45 negative population should be positioned on the far left of the histogram. The first marker is placed on the origin, the second marker is placed immediately to the right of the portion of the histogram showing the negative population, and subtraction is performed to calculate the number and percentage of non-white cells in the specimen. The following description outlines the above procedure with reference to the preferred flow cytometer and software, the FACScan running the Consort 30 program:

a. Select Consort 30 from main menu on FACScan.

b. Calibrate FSC (forward scatter), SSC (side scatter), and FL1 and FL2 (flourescence) settings.

c. Acquire and store data (press 2).

d. Place tube on cytometer.

e. Acquire data into memory (press 5).

f. Select "histogram" (press 1).

g. Exit when collection is complete (press 0).

NOTE: Since the data has not been saved, it is essential to continue with data analysis. Failure to do so will result in loss of data and the need to recollect data from the tube.

h. Analyze data (press 4).

i. Select single histogram (press 2).

j. Change parameter to FL-1 (press <shift>–1).

k. Set markers (press <shift>–3).

l. Number markers (press 2).

The CD45 negative population should be positioned on the far left of the histogram. Set marker 1 on the origin by pressing the <return> key without moving the marker. Set marker 2 immediately to the right of the negative histogram.

m. Press any key and then press <shift> 9 to print the CD45 negative count, i.e., the number of non-white cells in the specimen.

The CD45 negative population does not necessarily represent tumor cells. Normal epithelial cells, rather than tumor cells, may still be represented within this population.

It is understood that other methods known to the art for measuring the monocyte population may be used to compensate the flow cytometer, and that the method is not limited to the use of FITC-labeled CD45 MAb. As an example, another preferred detection agent is anti-cytokeratin bound to FITC or to other fluorochromes known to the art.

(10) STAINING

The drug and control tubes are removed from the incubator and centrifuged at high speed (Sero-fuge II) for 3 minutes. The supernatant is removed and the cell button is resuspended by gentle vortexing.

If the percentage of CD45-negative cells, i.e., non-white cells, is less than 80%, steps must be taken to set the compensation on the flow cytometer. 10.0 µl of FITC-labeled CD45 MAb is added to one of the negative control samples and the sample is incubated at room temperature for 15 minutes. The sample is washed once by adding 2.0 ml of PBS to the tube, centrifuging at high speed, and decanting the supernatant, and is then washed a second time by the same procedure.

0.5 ml of 0.05 mg/ml propidium iodide reagent is added to each drug sample and non-FITC-CD45 labeled control sample. All samples are then allowed to stand for a minimum of 10 minutes at room temperature. The samples should not be left for more than one hour after the addition of propidium iodide reagent.

(11) DATA ACQUISITION

The flow cytometer is then used to analyze the drug and control samples. Following is an outline of the preferred mode of flow cytometric analysis.

The FSC (forward scatter) and SSC (90-degree or side scatter) settings are placed in linear mode. The FL-1 and FL-2 (fluorescence scales) are placed in logarithmic mode. 5000 acquisition samples are preferred. A negative control tube is placed on the cytometer, a dot-plot is generated, and the FSC and SSC controls are adjusted to position the major cell population(s) in the center of the plot. The negative gate is set in the FL-2 histogram to exclude the negative control population, and this gate is stored for use in the analysis of the drug samples.

If double staining with propidium iodide reagent and CD45-FITC is used due to a non-white cell content of below 80%, additional compensation adjustments must also be

made. The CD45-FITC labeled control sample is placed in the flow cytometer and the steps noted above are repeated to produce and store a second negative gate for the CD45 positive subpopulation so that these white cells may later be excluded when analyzing the drug samples.

The data editing function of the flow cytometer is then accessed and each drug sample is analyzed with the control populations excluded. Data is stored for each control and drug sample.

When using the preferred flow cytometer and software, the FACScan running the Consort 30 program, the procedure listed above is performed as follows:

(a) Select CONSORT 30 from the main menu using the mouse.

(b) Insert the data storage disk into the disk drive.

(c) Accept date and time if correct by pressing Y. Settings should be linear on FSC and SSC and logarithmic on FL-1 and FL-2. Adjust as necessary.

(d) Select "acquire and store data" (press 2). The menu will appear.

(e) Select "storage file name" (press 7).

(f) Select "acquisition events" (press 2). Collect 5000 events.

(g) Select "acquire data into memory" (press 5).

(b) Place the control tube on the cytometer, select dot-plot and use the FSC and SSC controls to position the major cell population(s) in the center of the plot. Set FSC threshold as necessary.

NOTE: If double staining with PI and CD45-FITC is used, FL1-FL2 compensation must also be performed by use of the CD45-FITC negative control sample noted above at (10).

(i) Change to FL-2 histogram and use the FL-2 detector control to set the negative gate.

(j) Exit to menu (press 0).

(k) Select "edit text" (press 4).

(l) Enter control sample or drug sample name, patient name, etc. and other such data as desired.

(m) Return to menu (press <cntrl>–0).

(n) Select "acquire and save data" (press <shift>–5) to collect 5000 events.

(o) Repeat steps (k)–(n) until data for all tubes have been collected.

(p) Select "quit program" (press 0) to return to main menu.

(12) DATA ANALYSIS

The flow cytometric analysis software is then used to calculate the percent viability of the positive control and drug samples and compile the viability results of these samples into tabular form. The following steps detail this procedure for the preferred flow cytometer, the FACScan running the Consort 30 program:

(a) Select "read file from disk" (press 3).

(b) Enter file name in correct format starting with tag 1 (control).

(c) Select "read file from disk" (press 2).

(d) Exit (press 0).

(e) Select "analyze data" (press 4).

(f) Select "single histogram" (press 2).

(g) Select "parameter" (<shift>–1 for FL-2).

(h) Select "set markers" (press <shift>–3). Select 2 markers, one at the origin (press ENTER) and the second at the bottom of the negative peak.

(i) Select "display statistics" (press 9) and press "Y" to save data in file.

(j) Obtain hard copy of results (press <shift>–9).

(k) Exit (press 0).

(l) Exit again (press 0) and save the file (enter 14).

(m) Repeat steps (a)–(f) and (i)–(l) until all data have been printed and saved. Do not adjust the markers once they have been set.

The results of the assay are considered to be acceptable if the viability of the positive control sample is less than 50% of the negative control sample(s). If the viability of the positive control sample is greater than 50% of the negative control sample, the results are discarded and a new assay is run.

FORMULATION OF A MULTIDRUG CHEMOTHERAPEUTIC REGIMEN

A multidrug chemotherapeutic regimen is then chosen for the patient based on the results of the pharmacosensitivity assay performed on the patient's tumor. A tumor may be considered to have high sensitivity to a particular drug if greater than 75% of the viable tumor cells are shown to be apoptotic; medium sensitivity if approximately 30–75% of the viable cells are apoptotic; low sensitivity if approximately 15%–30% of the viable tumor cells are apoptotic; and drug resistant if less than 15% of the viable tumor cells are apoptotic. Sensitivity may be rapidly calculated by, for example, custom software or spreadsheet programs.

In general, the multidrug treatment regimen is formulated by selecting the drugs for which the tumor exhibits the highest pharmacosensitivity. Usually, no more than ten drugs are chosen per regimen, with four of these being biological response modifiers (and generally one of these being a hormone). Another preferred treatment is to apply the four or five most effective non-biological response modifier drugs plus one or two biological response modifiers. Treatments wherein four non-biological response modifier drugs plus an alpha interferon (and occasionally an additional biological response modifier, generally a hormone) have been tested with excellent results, some of which are summarized below.

However, it must be remembered that selection criteria based on sensitivity levels will not always formulate the best multidrug treatment regimen. Certain drugs must be eliminated, or dosages must be reduced, if the toxicity of the assay-recommended treatment regimen is too high. Also, the pharmacosensitivity assay results must be interpreted with caution, as the clinical significance of the sensitivity levels has not been established for all drugs in the assay. As examples, levamisole, megestrol acetate, and retinoic acid are not cytotoxic drugs, and tumor cell viability in response to these drugs does not differ significantly from control cells. Therefore, a low level of tumor cell response to these drugs should not be interpreted as drug resistance.

ADMINISTRATION OF THE REGIMEN

The multidrug regimen may be administered by any of the following methods known to the art, such as intravenous administration or even oral administration (where appropriate). The drugs are generally administered at the maximum single-agent dosage recommended by the manufacturer. Dosages are reduced if the combined organ toxicity or the cumulative dosage toxicity of the multidrug regimen is irreversible. Other factors such as the time interval between prior surgery and present chemotherapy and the patient's present status may indicate that lesser dosages should be used.

To insure that the in vivo results of the patient's treatment regimen track the in vitro pharmacosensitivity assay results as closely as possible, the same drug stock is used for both the assay drug samples and the treatment regimen.

One preferred method of drug administration is locoregional administration, in addition to (or in lieu of) systemic administration. Chemotherapy for solid tumors in man is usually given systemically, either orally or intravenously. Consequently, the antitumor effect (the drug level achieved at the target) depends on the blood supply of the solid tumor. Since such blood supply is often small, reducing the delivery of the drug to the tumor, the use of locoregional intra-arterial infusion or perfusion chemotherapy has recently been of interest. Locoregional intra-arterial therapy involves the introduction of a percutaneous (i.e., through the skin) catheter into an artery that feeds the tumor. Chemotherapeutic drugs are then supplied through the catheter, with or without the use of a vasodilator to increase the blood supply to the tumor. As an example, for liver tumors, a catheter would be introduced into the hepatic artery. (Note that while 80%–90% of the blood supply of the liver is derived from the portal vein, the blood supply of liver tumors almost exclusively tends to be from the hepatic artery.) An excellent review of intra-arterial therapy of primary and metasiatic disease may be found in Haskell et al., ed., Cancer Treatment, 3rd. ed., W. B. Saunders Company, 1990.

The theory underlying locoregional intra-arterial drug therapy is that tumor cells should be exposed to higher drug concentrations, and that locoregional administration provides effective delivery of cytotoxic drugs to the tumor in concentrations greater than could be obtained by systemic administration. By supplying the drugs directly to the artery that feeds the tumor, a five- to twenty-fold increase in drug concentration can be obtained in the tumor as compared to systemic therapy, with only minimal toxicity to other organs. Other organs are not exposed to the maximum drug dose because locoregional drug administration achieves high drug concentration only on the "first pass": after the drug passes through the target organ and becomes mixed with the venous blood, any further drug exposure experienced by non-target organs will be the same as though the drug had been given systemically via a peripheral vein. By allowing such dose escalation, marginally effective drugs can be used to increase cell kill, circumvent existing drug resistance mechanisms of the tumor, and prevent the emergence of new drug resistance patterns for the tumor.

However, certain drawbacks to locoregional intra-arterial administration limit its use. It is generally recognized that percutaneously placed angiographic catheters or surgically introduced stiff plastic catheters frequently result in progressive artery thrombosis with decreasing blood flow (or even complete occlusion), catheter displacement, and patient discomfort and inconvenience. Moreover, the often prolonged hospitalization required after percutaneous catheterization is a major obstacle. The catheters are also inconvenient due to the need for frequent maintenance by medical personnel. However, a number of technical developments have largely solved these problems and have greatly improved the feasibility of long-term arterial catheterization and intracavitary treatment.

First, the availability of flexible polymeric silicone (silastic) catheters for surgical placement, e.g., the Hickmann and the Tenckhoff catheter systems, has greatly reduced morbidity, and these systems can remain in place for over a year. For this reason, silastic catheters are preferred.

Second, the problems of maintenance and care have also been reduced by the introduction of portals which can be attached to the intracavitary or intra-arterial catheter and fixed in a subcutaneous pocket to allow repeated access to the peritoneal cavity or the blood vessel in question. As an example, a catheter can be placed in the hepatic artery via the gastroduodenal artery during laparotomy. It can then be threaded through a subcutaneous tunnel and attached to a vascular access port, which also is anchored subcutaneously, usually in the upper anterior rib cage. When a special "HUBER-POINT" (Exel International, Inc., Culver City, Calif., U.S.A.) needle is used, the portal can be punctured through the skin and the thick self-sealing portal septum as often as necessary. Chemotherapeutic agents are then pumped in by a small, external, battery-powered chemoinfusion pump the patient carries in a pouch, achieving continual intra-arterial chemoinfusions. The "PORT-A-CATH" (Harbor Medical Devices, Inc., Jaffrey, N.H., U.S.A.) catheter/portal systems have proven to function admirably well. Of these, the "PORT-A-CATH" is particularly preferred. Its large portal membrane size is of great advantage since the location of a very small subcutaneous membrane via a slippery and sometimes fluid-filled pocket may be troublesome, particularly in obese patients.

For outpatients, continuous drug infusion can be provided by a totally implantable subcutaneous pump, e.g. the "INFUSAID" pump (Infusaid Corp., Norwood, Mass.). The "INFUSAID" utilizes an expandable reservoir inside a smooth, rigid titanium external shell. Between the shell and the reservoir is a fluorocarbon liquid in equilibrium with its vapor phase, and a catheter is attached to the reservoir. During laparotomy, the pump is placed in a subcutaneous pocket, and the catheter is inserted into the hepatic artery. The reservoir, which holds 50 ml, is filled through a silastic port in the pump by insertion of a needle through the skin and subcutaneous tissue. The pressure of this injection expands the bellowslike reservoir, simultaneously filling the pump and condensing the fluorocarbon. Since at 37° C. the vapor pressure of the substance is 300 mm greater than atmospheric pressure, the heat of the body causes a phase change in the fluorocarbon, and the expanding vapor exerts force on the bellows, which forces the infusate through a flow-regulating resistance element and out the catheter. At flow rates of 2 to 3 ml per 24 hours, the pump can run for 14 to 21 days without refilling. Each refilling recharges the driving mechanism. The device is quiet and efficient and has proved to be virtually free of mechanical or technical problems.

There are several significant advantages to totally implantable subcutaneous pumps. Patient acceptance has been enthusiastic since all forms of activity, including recreational activities such as tennis, swimming, and golf, are unimpaired. Filling the pump has proved to be a relatively quick and painless procedure, and the scarification of veins common with intravenous administration of therapeutic agents is obviated. Most important, because the entire system is internalized and because of the unique thick-walled, small-diameter lumen design of the catheter of the "INFUSAID" system, catheter occlusion and migration have been virtually unknown. With these technical improvements, long-term periods of continuous infusion can be accomplished. This technology has largely eliminated the vagaries of external catheter occlusions, displacement, inadvertent withdrawal, and similar technical problems that have plagued hepatic artery infusion chemotherapy in the past.

Another alternative is an extracorporeal pumping device such as the "ACT-A-PUMP" (Pharmacia, Dublin, Ohio, U.S.A.). Extracorporeal pumps are economical since they may be used for more than one patient, but they tend to be

more inconvenient to the patient because the patient requires a continuous percutaneous needle connection to an external pump.

The procedure of the invention has achieved exceptionally favorable results in the treatment of metastatic tumors, particularly metasiatic liver tumors, by use of locoregional infusion. Generally, metastatic tumors are treated with the same regimen used for treating a primary tumor. As numerous studies in the art illustrate, this often results in the defeat of the primary tumor only to have the metastases overwhelm the patient; a battle is won, but the war is lost. It has been found that where the drug treatment regimen of the procedure is developed by pharmacosensitivity testing of a metastatic tumor sample, the regimen is highly effective not only on the metastases, but on the primary tumor as well. The metastases are treated by locoregional infusion, while the primary tumor may be treated simultaneously by systemic administration (or can also be treated locoregionally, if appropriate). When the primary tumor is no longer present, the locoregional treatment of the metastases may be continued; alternatively, if the metastases are eliminated, the systemic treatment of the primary tumor may continue. The results of treatment by simultaneous locoregional and systemic administration of chemotherapy dictated by testing of metastases are given in Experiments 29–33 below. The classical oncologist might view the treatment applied in these experiments to be an aggressive approach, but it is believed that any inconveniences presented by the use of locoregional therapy are outweighed by the fact that the procedure offers far better response rates than prior art treatments.

The three methods of locoregional intra-arterial administration used most often in the procedure have been:

(1) Super-selective catheterization wherein the catheter is inserted in the tumor nutrition artery. Generally, drugs are injected one to three times a day for 3 to 7 days.

(2) The catheter is placed in the tumor nutrition artery, generally once per week, and after drugs are administered it is removed. This procedure is repeated, generally until remission.

(3) A permanent catheter is surgically implanted and the drugs are administered by continuous infusion. All three methods have met with positive results with minimal ill effects in the patient.

Before the catheter is installed, the patient's vascular system should be carefully examined because insufficient knowledge about vascular anomalies can result in non-optimal infusion of the target organ. Ligation of vessels and the use of more than one catheter may be necessary to obtain optimal infusion of the organ.

It must be remembered that while many drugs can be applied either systemically or locoregionally, not all drugs are appropriate for all types of administration. As one example, mechlorethamine hydrochloride (nitrogen mustard) is too toxic to veins to be applied locoregionally. As another example, many of the drugs would either be ineffective or deadly if applied orally. The manufacturer's instructions for use should be carefully followed in every case. Additionally, if locoregional and systemic administration are used simultaneously, dosages must be adjusted to account for overall toxicity. When the dosages of the systemically-applied drugs are adjusted to account for the locoregionally-administered dosages, simultaneous locoregional and systemic treatment is actually much less toxic than general systemic therapy alone. In some cases, depending on the drug take-up of the target organ, it may be possible to forego systemic administration in lieu of locoregional administration because the venous blood exiting the target organ may carry the dosages appropriate for systemic therapy.

EXPERIMENTAL RESULTS

Following is a summary of experimental results obtained by use of the procedure described above. Experiments 1–28 were performed to determine the variation in drug sensitivity between patient tumors as determined by the pharmacosensitivity assay of the procedure, and these experiments demonstrated that the same and similar types of tumors vary widely in in vitro response to the same drugs from patient to patient. Experiments 29–33 tested the efficacy of the treatment procedure on candidates with primary liver tumors and liver metastases, wherein the drugs were administered intra-arterially to the liver via the hepatic artery.

Experiment 1: Patient (1) suffered from pancreatic cancer. A tumor sample was tested by the preferred embodiment of the pharmacosensitivity assay as set out above and the following results were obtained:

High Sensitivity	
Fludarabine phosphate	94.3%
Doxorubicin	93.1%
Dactinomycin	92.8%
Taxol	84.8%
Intermediate Sensitivity	
Etoposide	71.9%
Mito-C	68.1%
Thio-TEPA	52.1%
nHuIFNπ	49.5%
VBL	37.3%
Ifosfamide/Mesna	35.5%
Low Sensitivity	
5-FU	31.7%
Bleomycin	31.1%
nHuIFNα	30.6%
rIFNβ-1b	28.3%
Cyclophosphamide	27.4%
rIFNτ-1b	26.7%
Prednisone	26.5%
GM-CSF	25.7%
Ifosfamide	22.7%
VCR	22.3%
Resistant	
Paraplatin	19.7%
rIFNα-2a	13.2%
Floxuridine	12.9%
nHuIFNβ	12.4%
rIL-2	8.8%
Cisplatin	8.7%
rTNF	7.5%
Amethopterin	6.4%
Retinoic acid	3.5%
G-CSF	3.4%
Hydrea	2.9%
Megestrol Acetate	2.1%
Tamoxifen	0.0%

The positive (Abrin) control sample demonstrated 99.6% cell kill as compared to the negative control sample.

Experiment 2: Patient (2) suffered from pancreatic cancer. A tumor sample was tested by the preferred embodiment of the pharmacosensitivity assay as set out above and the following results were obtained:

High Sensitivity	
Dactinomycin	99.5%
Doxorubicin	98.0%
Tamoxifen	94.6%
Mito-C	92.1%
5-FU	89.8%
Intermediate Sensitivity	
Taxol	77.6%
Retinoic acid	64.8%
Etoposide	54.0%
G-CSF	51.7%
Amethopterin	50.2%
Ifosfamide	47.5%
Floxuridine	44.7%
rTNF	43.5%
rIFNα-2a	40.5%
Low Sensitivity	
Ifosfamide/Mesna	34.3%
rIFNβ-1b	34.2%
GM-CSF	32.3%
rIFNβ-1b	29.1%
rIFNτ-1b	28.2%
Medroxyprogesterone	25.6%
BCNU	25.1%
Megestrol Acetate	23.4%
rIL-2	21.2%
Streptozocin	21.2%
Resistant	
VCR	19.3%
VBL	9.6%
Somatostatin	0.0%
nHuIFNα	0.0%
nHuIFNπ	0.0%

The positive (Abrin) control sample demonstrated 99.7% cell kill as compared to the negative control sample.

Experiment 3: Patient (3) suffered from pancreatic cancer. A tumor sample was tested by the preferred embodiment of the pharmacosensitivity assay as set out above and the following results were obtained:

Intermediate Sensitivity	
G-CSF	53.5%
GM-CSF	53.5%
rIFNτ-1b	50.1%
Doxorubicin	43.9%
Mito-C	43.9%
rTNF	41.1%
nHuIFNβ	38.7%
Low Sensitivity	
nHuIFNπ	29.4%
Dactinomycin	27.3%
rIFNβ-1b	23.0%
VBL	22.3%
VCR	21.6%
Resistant	
Ifosfamide/Mesna	18.1%
rIL-2	17.7%
Ifosfamide	12/7%
BCNU	11.5%
Retinoic acid	11.1%
5-FU	10.9%
Amethopterin	9.7%
Etoposide	0.0%
Floxuridine	0.0%
Streptozocin	0.0%
Taxol	0.0%
Megestrol Acetate	0.0%
Medroxyprogesterone	0.0%
Somatostatin	0.0%
Tamoxifen	0.0%
nHuIFNα	0.0%
rIFNα-2a	0.0%

The positive (Abrin) control sample demonstrated 66.5% cell kill as compared to the negative control sample.

Experiment 4: Patient (4) suffered from pancreatic cancer. A tumor sample was tested by the preferred embodiment of the pharmacosensitivity assay as set out above and the following results were obtained:

High Sensitivity	
Dactinomycin	86.9%
Doxorubicin	83.9%
Intermediate Sensitivity	
Medroxyprogesterone	71.0%
rIFNα-2a	48.7%
Mito-C	48.2%
G-CSF	47.1%
nHuIFNπ	46.9%
GM-CSF	41.3%
Megestrol Acetate	39.8%
rIFNβ-1b	38.3%
Low Sensitivity	
rTNF	33.5%
Tamoxifen	32.2%
5-FU	31.7%
nHuIFNβ	30.6%
rIFNτ-1b	24.4%
VCR	21.3%
Retinoic acid	20.3%
Resistant	
Floxuridine	8.0%
VBL	7.6%
BCNU	4.6%
Streptozocin	3.2%
Ifosfamide/Mesna	1.3%
Amethopterin	0.2%
Etoposide	0.0%
Ifosfamide	0.0%
Taxol	0.0%
Somatostatin	0.0%
nHuIFNα	0.0%
rIL-2	0.0%

The positive (Abrin) control sample demonstrated 31.7% cell kill as compared to the negative control sample.

Experiment 5: Patient (5) suffered from pancreatic cancer. A tumor sample was tested by the preferred embodiment of the pharmacosensitivity assay as set out above and the following results were obtained:

High Sensitivity	
Doxorubicin	98.5%
Dactinomycin	95.6%
Intermediate Sensitivity	
Mito-C	73.8%
VBL	39.1%
VCR	35.3%
Low Sensitivity	
BCNU	23.5%

-continued

Resistant	
Retinoic acid	17.8%
5-FU	15.2%
Tamoxifen	14.8%
Streptozocin	12.8%
Medroxyprogesterone	11.6%
Somatostatin	7.8%
Ifosfamide/Mesna	7.7%
rIL-2	5.5%
nHuIFNα	2.7%
Floxuridine	2.5%
Taxol	0.7%
Etoposide	0.0%
Ifosfamide	0.0%
Amethopterin	0.0%
G-CSF	0.0%
GM-CSF	0.0%
Megestrol Acetate	0.0%
nHuIFNβ	0.0%
nHuIFNπ	0.0%
rIFNα-2a	0.0%
rIFNβ-1b	0.0%
rIFNτ-1b	0.0%
rTNF	0.0%

The positive (Abrin) control sample demonstrated 88.1% cell kill as compared to the negative control sample.

Experiment 6: Patient (6) suffered from pancreatic cancer. A tumor sample was tested by the preferred embodiment of the pharmacosensitivity assay as set out above and the following results were obtained:

High Sensitivity	
Doxorubicin	99.8%
Medroxyprogesterone	96.0%
Dactinomycin	94.7%
Tamoxifen	86.3%
Mito-C	81.5%
Intermediate Sensitivity	
Taxol	77.2%
Etoposide	67.4%
BCNU	61.9%
rIFNτ-1b	48.0%
VBL	47.5%
nHuIFNα	46.8%
Megestrol Acetate	46.6%
rTNF	45.7%
VCR	45.4%
G-CSF	45.4%
GM-CSF	44.6%
nHuIFNβ	39.1%
Low Sensitivity	
nHuIFNπ	34.9%
5-FU	25.0%
Resistant	
rIL-2	17.0%
Ifosfamide	7.4%
Streptozocin	5.4%
rIFNβ-1b	2.8%
Amethopterin	1.3%
Floxuridine	0.0%
Ifosfamide/Mesna	0.0%
Somatostatin	0.0%
rIFNα-2a	0.0%

The positive (Abrin) control sample demonstrated 98.7% cell kill as compared to the negative control sample.

Experiment 7: Patient (7) suffered from acute myelogenous leukemia. A tumor sample was tested by the preferred embodiment of the pharmacosensitivity assay as set out above and the following results were obtained:

High Sensitivity	
Doxorubicin	99.7%
Ara-C	95.3%
Fludarabine phosphate	80.3%
Intermediate Sensitivity	
GM-CSF	77.6%
G-CSF	56.5%
nHuIFNπ	49.7%
Cyclophosphamide	45.2%
nHuIFNα	44.6%
Prednisone	44.0%
Hydrea	43.9%
rIFNτ-1b	43.6%
rTNF	41.5%
nHuIFNβ	38.7%
Resistant	
rIFNβ-1b	16.3%
Amethopterin	13.6%
rIFNα-2a	10.8%
Cladribine	0.0%
rIL-2	0.0%

The positive (Abrin) control sample demonstrated 98.8% cell kill as compared to the negative control sample.

Experiment 8: Patient (8) suffered from breast cancer. A tumor sample was tested by the preferred embodiment of the pharmacosensitivity assay as set out above and the following results were obtained:

Intermediate Sensitivity	
Taxol	68.3%
nHuIFNβ	62.1%
nHuIFNπ	61.7%
GM-CSF	60.2%
Doxorubicin	56.5%
rIFNτ-1b	55.7%
rTNF	55.1%
Ara-C	48.7%
nHuIFNα	48.1%
G-CSF	44.4%
Medroxyprogesterone	43.1%
Melphalan	41.9%
Etoposide	36.6%
Low Sensitivity	
Ifosfamide	33.7%
Ifosfamide/Mesna	31.4%
Thio-TEPA	29.4%
rIFNα-2a	24.4%
Mito-C	22.6%
VBL	22.1%
Resistant	
VCR	19.4%
Prednisone	17.3%
rIFNβ-1b	17.0%
Tamoxifen	15.8%
Hydrea	12.2%
Cisplatin	11.0%
5-FU	8.3%
Cyclophosphamide	7.3%
Amethopterin	5.1%
Paraplatin	3.8%
rIL-2	3.2%
Floxuridine	0.0%
DTIC	0.0%
Somatostatin	0.0%

The positive (Abrin) control sample demonstrated 99.7% cell kill as compared to the negative control sample.

Experiment 9: Patient (9) suffered from breast cancer with liver metastases. A tumor sample was tested by the preferred

embodiment of the pharmacosensitivity assay as set out above and the following results were obtained:

High Sensitivity	
Taxol	93.5%
rTNF	90.5%
rIFNτ-1b	89.7%
nHuIFNα	85.0%
nHuIFNβ	81.7%
nHuIFNπ	80.4%
Intermediate Sensitivity	
G-CSF	78.3%
GM-CSF	77.2%
Doxorubicin	46.1%
Resistant	
Tamoxifen	13.1%
Etoposide	12.2%
BCNU	7.6%
Megestrol Acetate	3.6%
Medroxyprogesterone	2.4%
Ara-C	2.0%
Prednisone	1.5%
MITO-C	0.9%
5-FU	0.7%
Ifosfamide-Mesna	0.6%
VBL	0.3%
Paraplatin	0.0%
Cisplatin	0.0%
Cyclophosphamide	0.0%
Floxuridine	0.0%
Ifosfamide	0.0%
Amethopterin	0.0%
VCR	0.0%
Somatostatin	0.0%
rIL-2	0.0%
rIFNα-2a	0.0%
rIFNβ-1b	0.0%

The positive (Abrin) control sample demonstrated 75.0% cell kill as compared to the negative control sample.

Experiment 10: Patient (10) suffered from breast cancer. A tumor sample was tested by the preferred embodiment of the pharmacosensitivity assay as set out above and the following results were obtained:

High Sensitivity	
Doxorubicin	93.7%
Intermediate Sensitivity	
VCR	57.9%
Megestrol Acetate	54.4%
Somatostatin	54.4%
Tamoxifen	51.6%
Cyclophosphamide	49.0%
Floxuridine	47.1%
Etoposide	44.0%
Ifosfamide/Mesna	42.1%
BCNU	42.0%
nHuIFNπ	40.5%
Low Sensitivity	
Cisplatin	39.9%
Prednisone	37.0%
Ifosfamide	35.1%
VBL	33.9%
5-FU	29.2%
Paraplatin	25.6%
Taxol	21.5%
Resistant	
Ara-C	19.3%
Amethopterin	18.7%
Mito-C	18.1%
rIFNα-2a	18.0%
rIFNβ-1b	17.5%
rTNF	3.5%
G-CSF	0.0%
GM-CSF	0.0%
Medroxyprogesterone	0.0%
nHuIFNα	0.0%
nHuIFNβ	0.0%
rIFNτ-1b	0.0%

The positive (Abrin) control sample demonstrated 52.8% cell kill as compared to the negative control sample.

Experiment 11: Patient (11) suffered from bladder cancer. A tumor sample was tested by the preferred embodiment of the pharmacosensitivity assay as set out above and the following results were obtained:

Intermediate Sensitivity	
Bleomycin	77.3%
rIFNα-2a	69.2%
rIFNτ-1b	61.1%
Mito-C	58.3%
rIFNβ-1b	43.1%
Hydrea	42.2%
Low Sensitivity	
Retinoic acid	34.6%
Floxuridine	29.4%
Amethopterin	24.2%

The positive (Abrin) control sample demonstrated 97.6% cell kill as compared to the negative control sample.

Experiment 12: Patient (12) suffered from bladder cancer. A tumor sample was tested by the preferred embodiment of the pharmacosensitivity assay as set out above and the following results were obtained:

Intermediate Sensitivity	
Bleomycin	77.3%
rIFNα-2a	69.2%
rIFNτ-1b	61.1%
Mito-C	58.3%
rIFNβ-1b	43.1%
Hydrea	42.2%
Low Sensitivity	
Retinoic acid	34.6%
Floxuridine	29.4%
Amethopterin	24.2%

The positive (Abrin) control sample demonstrated 97.6% cell kill as compared to the negative control sample.

Experiment 13: Patient (13) suffered from chronic myelogenous leukemia. A tumor sample was tested by the preferred embodiment of the pharmacosensitivity assay as set out above and the following results were obtained:

High Sensitivity	
Doxorubicin	94.0%
Fludarabine phosphate	81.0%
Ara-C	80.6%

-continued

Intermediate Sensitivity	
Hydrea	50.1%
Cyclophosphamide	38.9%
Resistant	
nHuIFNβ	19.1%
nHuIFNπ	15.1%
rTNF	13.5%
Amethopterin	12.2%
Prednisone	9.0%
rIFNτ-1b	8.9%
rIFNβ-1b	8.3%
rIFNα-2a	7.9%
G-CSF	0.0%
GM-CSF	0.0%
Somatostatin	0.0%
nHuIFNα	0.0%
rIL-2	0.0%

The positive (Abrin) control sample demonstrated 99.6% cell kill as compared to the negative control sample.

Experiment 14: Patient (14) suffered from chronic myelogenous leukemia. A tumor sample was tested by the preferred embodiment of the pharmacosensitivity assay as set out above and the following results were obtained:

Intermediate Sensitivity	
Fludarabine phosphate	65.1%
Ara-C	60.1%
Cladribine	58.3%
rIFNβ-1b	49.2%
Doxorubicin	42.9%
nHuIFNβ	40.3%
Low Sensitivity	
Hydrea	32.7%
rIFNα-2a	30.3%
Prednisone	27.8%
Amethopterin	26.9%
nHuIFNπ	24.9%
Cyclophosphamide	23.3%
rIFNτ-1b	23.0%
Resistant	
nHuIFNα	18.3%
rTNF	14.1%
rIL-2	13.9%
Etoposide	11.1%
Somatostatin	8.1%
G-CSF	0.0%
GM-CSF	0.0%

The positive (Abrin) control sample demonstrated 99.6% cell kill as compared to the negative control sample.

Experiment 15: Patient (15) suffered from chronic myelogenous leukemia. A tumor sample was tested by the preferred embodiment of the pharmacosensitivity assay as set out above and the following results were obtained:

High Sensitivity	
Doxorubicin	99.9%
Intermediate Sensitivity	
G-CSF	60.4%
Hydrea	52.0%
Ara-C	47.1%
Fludarabine phosphate	46.3%
Cladribine	44.3%

-continued

GM-CSF	41.7%
rIFNβ-1b	40.2%
nHuIFNπ	35.4%
Low Sensitivity	
nHuIFNβ	27.3%
nHuIFNα	25.8%
rTNF	24.0%
rIFNα-2a	23.7%
Resistant	
rIFNτ-1b	20.0%
Amethopterin	10.5%
Cyclophosphamide	3.8%
Prednisone	2.2%
Somatostatin	0.0%
rIL-2	0.0%

The positive (Abrin) control sample demonstrated 99.7% cell kill as compared to the negative control sample.

Experiment 16: Patient (16) suffered from chronic myelogenous leukemia. A tumor sample was tested by the preferred embodiment of the pharmacosensitivity assay as set out above and the following results were obtained:

High Sensitivity	
Doxorubicin	94.0%
Fludarabine phosphate	81.0%
Ara-C	80.6%
Intermediate Sensitivity	
Hydrea	50.1%
Cyclophosphamide	38.9%
Resistant	
nHuIFNβ	19.1%
nHuIFNπ	15.1%
rTNF	13.5%
Amethopterin	12.2%
Prednisone	9.0%
rIFNτ-1b	8.9%
rIFNβ-1b	8.3%
rIFNα-2a	7.9%
G-CSF	0.0%
GM-CSF	0.0%
Somatostatin	0.0%
nHuIFNα	0.0%
rIL-2	0.0%

The positive (Abrin) control sample demonstrated 99.6% cell kill as compared to the negative control sample.

Experiment 17: Patient (17) suffered from chronic myelogenous leukemia. A tumor sample was tested by the preferred embodiment of the pharmacosensitivity assay as set out above and the following results were obtained:

High Sensitivity	
Doxorubicin	99.8%
Fludarabine phosphate	98.8%
Ara-C	98.2%
Prednisone	82.2%
Intermediate Sensitivity	
Cyclophosphamide	53.6%
Hydrea	45.7%
rIFNα-2a	43.4%
rIFNτ-1b	42.3%
nHuIFNβ	39.5%
nHuIFNπ	39.3%

-continued

rIFNβ-1b	35.6%
Low Sensitivity	
nHuIFNα	33.3%
rTNF	25.6%
G-CSF	21.0%
Resistant	
Amethopterin	15.2%
Somatostatin	14.1%
rIL-2	5.5%
GM-CSF	0.0%

The positive (Abrin) control sample demonstrated 97.9% cell kill as compared to the negative control sample.

Experiment 18: Patient (18) suffered from chronic myelogenous leukemia. A tumor sample was tested by the preferred embodiment of the pharmacosensitivity assay as set out above and the following results were obtained:

High Sensitivity	
Doxorubicin	98.5%
Intermediate Sensitivity	
rTNF	66.8%
G-CSF	54.3%
Fludarabine phosphate	52.0%
nHuIFNπ	49.3%
Ara-C	46.7%
nHuIFNα	43.8%
rIFNτ-1b	43.6%
GM-CSF	39.7%
nHuIFNβ	36.8%
Cyclophosphamide	35.9%
Low Sensitivity	
Prednisone	31.1%
Resistant	
Hydrea	0.0%
Amethopterin	0.0%
Somatostatin	0.0%
rIL-2	0.0%
rIFNα-2a	0.0%
rIFNβ-1b	0.0%

The positive (Abrin) control sample demonstrated 97.9% cell kill as compared to the negative control sample.

Experiment 19: Patient (19) suffered from chronic myelogenous leukemia. A tumor sample was tested by the preferred embodiment of the pharmacosensitivity assay as set out above and the following results were obtained:

High Sensitivity	
Doxorubicin	99.1%
Intermediate Sensitivity	
Ara-C	69.8%
Fludarabine phosphate	59.5%
Low Sensitivity	
nHuIFNπ	34.7%
nHuIFNβ	28.9%
nHuIFNα	26.7%
Hydrea	26.6%
G-CSF	25.0%
Resistant	
Cyclophosphamide	18.2%

-continued

rTNF	16.8%
rIFNτ-1b	16.4%
GM-CSF	14.5%
Prednisone	12.7%
rIFNβ-1b	5.8%
Amethopterin	2.3%
Somatostatin	0.0%
rIL-2	0.0%
rIFNα-2a	0.0%

The positive (Abrin) control sample demonstrated 99.4% cell kill as compared to the negative control sample.

Experiment 20: Patient (20) suffered from Waldenstrom's macroglobulinemia. A tumor sample was tested by the preferred embodiment of the pharmacosensitivity assay as set out above and the following results were obtained:

High Sensitivity	
rIFNτ-1b	91.8%
Intermediate Sensitivity	
nHuIFNπ	58.4%
rTNF	48.2%
G-CSF	46.7%
nHuIFNβ	42.1%
Fludarabine phosphate	36.4%
nHuIFNα	35.6%
Low Sensitivity	
GM-CSF	33.7%
Etoposide	31.6%
Medroxyprogesterone	30.9%
Ara-C	27.4%
rIFNα-2a	25.9%
Melphalan	23.2%
rIFNβ-1b	21.0%
Resistant	
Cladribine	17.2%
5-FLUOROURACIL	16.6%
Ifosfamide	14.8%
Cyclophosphamide	14.6%
Tamoxifen	11.2%
VCR	8.4%
Ifosfamide/Mesna	7.6%
VBL	5.2%
Doxorubicin	3.7%
Somatostatin	0.5%
Cisplatin	0.0%
rIL-2	0.0%

The positive (Abrin) control sample demonstrated 98.5% cell kill as compared to the negative control sample.

Experiment 21: Patient (21) suffered from Waldenstrom's macroglobulinemia. A tumor sample was tested by the preferred embodiment of the pharmacosensitivity assay as set out above and the following results were obtained:

Intermediate Sensitivity	
Doxorubicin	60.5%
Ara-C	35.6%
Low Sensitivity	
Fludarabine phosphate	30.2%
Mito-C	23.9%
Ifosfamide/Mesna	21.6%
Resistant	
rIFNβ-1b	10.1%

-continued

rIFNα-2a	8.8%
nHuIFNπ	4.1%
nHuIFNβ	3.5%
nHuIFNα	3.3%
Cyclophosphamide	1.7%
Cisplatin	0.0%
Etoposide	0.0%
5-FU	0.0%
Melphalan	0.0%
VBL	0.0%
VCR	0.0%
G-CSF	0.0%
GM-CSF	0.0%
Medroxyprogesterone	0.0%
Somatostatin	0.0%
Tamoxifen	0.0%
rIL-2	0.0%
rIFNτ-1b	0.0%
rTNF	0.0%

The positive (Abrin) control sample demonstrated 91.6% cell kill as compared to the negative control sample.

Experiment 22: Patient (22) suffered from Waldenstrom's macroglobulinemia. A tumor sample was tested by the preferred embodiment of the pharmacosensitivity assay as set out above and the following results were obtained:

High Sensitivity	
Doxorubicin	98.1%
Intermediate Sensitivity	
Fludarabine phosphate	50.4%
Ara-C	41.7%
Medroxyprogesterone	39.3%
Low Sensitivity	
Mito-C	22.5%
Ifosfamide/Mesna	20.1%
Resistant	
Cyclophosphamide	15.3%
VBL	9.4%
Tamoxifen	6.2%
Ifosfamide	3.1%
Cisplatin	2.8%
rIL-2	2.2%
nHuIFNα	1.1%
Etoposide	0.0%
5-FU	0.0%
Melphalan	0.0%
VCR	0.0%
G-CSF	0.0%
GM-CSF	0.0%
Somatostatin	0.0%
nHuIFNβ	0.0%
nHuIFNπ	0.0%
rIFNα-2a	0.0%
rIFNβ-1b	0.0%
rIFNτ-1b	0.0%
rTNF	0.0%

The positive (Abrin) control sample demonstrated 99.0% cell kill as compared to the negative control sample.

Experiment 23: Patient (23) suffered from non-Hodgkin's lymphoma. A tumor sample was tested by the preferred embodiment of the pharmacosensitivity assay as set out above and the following results were obtained:

Intermediate Sensitivity	
Fludarabine phosphate	36.4%
Low Sensitivity	
rTNF	21.1%
Ifosfamide	21.0%
Resistant	
Etoposide	20.0%
Doxorubicin	11.1%
Cyclophosphamide	10.0%
nHuIFNπ	8.9%
BCNU	7.7%
rIFNτ-1b	5.4%
VBL	5.2%
Ifosfamide/Mesna	5.1%
rIFNβ-1b	3.0%
Mechlorethamine hydrochloride	1.0%
nHuIFNβ	0.3%
Bleomycin	0.0%
DTIC	0.0%
Hydrea	0.0%
Amethopterin	0.0%
Streptozocin	0.0%
VCR	0.0%
Somatostatin	0.0%
nHuIFNα	0.0%
rIFNα-2a	0.0%
rIL-3	0.0%
rIL-2	0.0%

The positive (Abrin) control sample demonstrated 96.8% cell kill as compared to the negative control sample.

Experiment 24: Patient (24) suffered from osteogenic sarcoma. A tumor sample was tested by the preferred embodiment of the pharmacosensitivity assay as set out above and the following results were obtained:

High Sensitivity	
Doxorubicin	99.8%
Ara-C	97.7%
Intermediate Sensitivity	
Mito-C	59.6%
rIFNβ-1b	44.6%
Resistant	
5-FU	20.0%
Cisplatin	17.0%
Etoposide	16.6%
nHuIFNπ	12.5%
rIL-2	9.9%
DTIC	8.4%
Floxuridine	5.7%
nHuIFNβ	5.4%
Amethopterin	3.3%
Bleomycin	0.0%
Cyclophosphamide	0.0%
Ifosfamide	0.0%
Ifosfamide/Mesna	0.0%
Melphalan	0.0%
VBL	0.0%
VCR	0.0%
G-CSF	0.0%
GM-CSF	0.0%
Somatostatin	0.0%
nHuIFNα	0.0%
rIFNα-2a	0.0%
rIFNτ-1b	0.0%
rTNF	0.0%

The positive (Abrin) control sample demonstrated 99.6% cell kill as compared to the negative control sample.

Experiment 25: Patient (25) suffered from ovarian carcinoma. A tumor sample was tested by the preferred embodiment of the pharmacosensitivity assay as set out above and the following results were obtained:

High Sensitivity	
Doxorubicin	86.7%
Intermediate Sensitivity	
Ifosfamide	74.9%
rIFNβ-1b	72.5%
G-CSF	68.2%
GM-CSF	66.8%
Ara-C	66.4%
rIFNτ-1b	63.5%
rTNF	62.6%
Taxol	62.1%
nHuIFNα	60.7%
nHuIFNπ	60.2%
Mito-C	55.5%
5-FU	51.7%
Floxuridine	46.4%
VBL	43.6%
VCR	44.1%
Tamoxifen	41.7%
Megestrol Acetate	40.8%
Somatostatin	40.8%
Cyclophosphamide	39.8%
Amethopterin	39.3%
Low Sensitivity	
rIFNα-2a	34.1%
Resistant	
DTIC	14.2%
nHuIFNβ	7.6%
BCNU	0.0%
Cisplatin	0.0%
Etoposide	0.0%
Medroxyprogesterone	0.0%
rIL-2	0.0%

The positive (Abrin) control sample demonstrated 78.7% cell kill as compared to the negative control sample.

Experiment 26: Patient (26) suffered from glioblastima multiforma. A tumor sample was tested by the preferred embodiment of the pharmacosensitivity assay as set out above and the following results were obtained:

High Sensitivity	
nHuIFNβ	95.3%
rIFNτ-1b	95.3%
GM-CSF	94.7%
G-CSF	89.5%
rTNF	80.6%
Intermediate Sensitivity	
nHuIFNπ	79.8%
rIFNβ-1b	73.6%
Resistant	
Doxorubicin	8.6%
Ara-C	8.0%
Prednisone	7.2%
BCNU	5.6%
rIFNα-2a	5.4%
Cyclophosphamide	1.6%
Retinoic acid	0.0%
Cisplatin	0.0%
5-FU	0.0%
Amethopterin	0.0%
VCR	0.0%
Somatostatin	0.0%
nHuIFNα	0.0%
rIL-2	0.0%

The positive (Abrin) control sample demonstrated 91.0% cell kill as compared to the negative control sample.

Experiment 27: Patient (27) suffered from pleomorphic adenocarcinoma. A tumor sample was tested by the preferred embodiment of the pharmacosensitivity assay as set out above and the following results were obtained:

High Sensitivity	
nHuIFNα	81.6%
Intermediate Sensitivity	
nHuIFNβ	78.4%
nHuIFNπ	77.9%
rTNF	77.4%
G-CSF	76.5%
GM-CSF	72.4%
Doxorubicin	53.9%
Low Sensitivity	
BCNU	25.0%
Resistant	
Amethopterin	11.0%
Prednisone	6.3%
Ifosfamide	5.0%
Mito-C	3.1%
VCR	3.0%
Cyclophosphamide	2.5%
Dactinomycin	2.3%
Fludarabine phosphate	2.0%
rIFNβ-1b	3.4%
VBL	1.6%
Bleomycin	1.5%
DTIC	1.3%
Ifosfamide/Mesna	1.4%
rIFNα-2a	1.1%
rIFNτ-1b	0.5%
Ara-C	0.4%
Retinoic acid	0.0%
Hydrea	0.0%
Cisplatin	0.0%
5-FU	0.0%
Somatostatin	0.0%
rIL-2	0.0%

The positive (Abrin) control sample demonstrated 71.7% cell kill as compared to the negative control sample.

Experiment 29: Patient (28) suffered from melanoma. A tumor sample was tested by the preferred embodiment of the pharmacosensitivity assay as set out above and the following results were obtained:

Intermediate Sensitivity	
VBL	75.6%
Doxorubicin	72.1%
Ara-C	74.5%
Bleomycin	69.4%
Cyclophosphamide	67.3%
DTIC	55.9%
Mito-C	48.9%
Etoposide	47.5%
Taxol	43.2%
Tamoxifen	35.6%

-continued

Low Sensitivity	
G-CSF	34.0%
VCR	32.1%
Hydrea	31.5%
Mechlorethamine hydrochloride	26.7%
Ifosfamide/Mesna	26.0%
nHuIFNβ	24.8%
GM-CSF	24.5%
BCNU	23.0%
rIFNα-2a	22.3%
Fludarabine phosphate	21.8%
Cisplatin	20.9%
Ifosfamide	20.7%
Resistant	
nHuIFNπ	19.9%
rIFNβ-1b	19.9%
rIFNτ-1b	16.7%
Somatostatin	15.7%
rIL-2	15.0%
rTNF	12.8%
nHuIFNα	12.8%
Retinoic acid	0.5%
5-FU	0.0%

The positive (Abrin) control sample demonstrated 94.5% cell kill as compared to the negative control sample.

Experiment 29: 78 patients having liver metastases were treated by use of the procedure. The primary tumors were: breast 14; renal cell 8; colorectal 14; lung adenocarcinoma 12; melanoma 12; ovarian 8; primary liver 5; and salivary gland 5. All patients were previously unsuccessfully treated with conventional therapy. Tumor tissue was obtained by biopsy of the liver, and thus the metastases provided the basis for the pharmacosensitivity assay. A catheter was introduced percutaneously to the common hepatic artery or implanted during surgery. A combination of systemic and locoregional chemotherapy was administered 8 times for 3 hours every 4 weeks, with drugs and dosages chosen according to the assay. 43 drugs were tested with the assay, 5 of these being biological response modifiers. All 78 patients' tumors demonstrated sensitivity to doxorubicin, mito-C, cisplatin and interferon alpha (IFNα). Patients with the following tumors also demonstrated sensitivity to the following drugs: carboplatin for ovarian and lung cancer; floxuridine for colon cancer; methotrexate for breast and salivary gland cancer; dacarbazine for melanoma; etoposide for renal cell and primary liver cancer; and bleomycin for salivary gland cancer. It is hypothesized that the correlation between the pharmacosensitivity of the metastases and the type of the primary tumor involved is a reflection of the particular weaknesses of the primary tumor cell lines.

After 4 courses of treatment, 23 patients achieved complete remission for 8 or more months; partial remission was achieved in 35 patients for up to 24 months; no change was achieved in 14 patients for 8 months; and 16 patients demonstrated disease progression. Side-effects of nausea, vomiting, leukopenia, anemia and thrombocytopenia were experienced, but all were transitory and of short duration. The experiment showed that the procedure offers an effective choice of drugs for overall treatment, i.e., simultaneous treatment of both primary tumors and (liver) metastases, with minimal side-effects.

Experiment 30: In 17 patients (average age 57 years, varying from 32–71 years; 6 females, 11 males) suffering from colon carcinoma with liver metastases, an intra-arterial, intra-hepatic catheter was implanted. Tumor tissue was obtained by liver biopsy during this procedure, and thus the liver tumors provided the basis for the pharmacosensitivity assay. During the assay, the tumor was tested with 26 drugs (including 5 biological response modifiers). Drug sensitivity to the same drugs varied considerably amongst the patients. The four most active drugs for each patient, plus interferon alpha (IFNα) were administered to each patient, and each drug was administered at the highest recommended dosage. Systemic chemotherapy was applied, and one-day locoregional infusion was administered every 4 weeks. Complete remission was obtained in 5 patients for at least 18 months, and three patients are still in complete remission after 3 years. Partial remission was obtained in 10 patients, varying in duration from 8 and 24 months. No change was obtained for 1 patient for 8 months. One patient showed disease progression. Minimal side-effects (nausea, vomiting, thrombocytopenia) were observed. The experiment showed that the procedure offers an effective choice of drugs for liver metastases of colon carcinoma when given in locoregional infusion, with minimal side-effects.

Experiment 31: 14 patients suffering from pancreatic cancer with extensive liver metastases were treated by use of the procedure (median age 56, age from 32–78, 2 females, 12 males). Liver tumor cells were tested with 30 drugs (including 6 biological response modifiers and 3 hormones). Doxorubicin, methotrexate, floxuridine, streptozotocin, BCNU, mito-C, cis-platinum, carboplatin, and interferons alpha and gamma (IFNα and IFNτ) were found to have activity in the pharmacosensitivity assay. A percutaneously introduced intra-arterial, intra-hepatic catheter was administered directly to the pancreas and the liver (with approximately 33% delivery to the pancreas and 67% to the liver). The four most active drugs for each patient and IFNα and IFNτ were used for the respective patient. Over 3 hours of locoregional infusion were administered every 4 weeks for 6 weeks along with systemic administration.

2 patients achieved complete remission for 18 or more months (with both patients being alive after 3 years). 8 patients achieved partial remission; 6 of these had radical surgery, 4 with complete resection. Partial remission was achieved for between 8 and 24 months. 1 patient achieved no change for 6 months. Minimal side effects were observed. The experiment showed that the procedure offers an effective choice of drugs for pancreatic cancer and liver metastases with minimal side-effects.

Experiment 32: 21 patients suffering from colon cancer with liver metastases were treated by the procedure (average age 61, from 35–75 years of age, 8 females, 13 males). All patients failed to respond to previous extensive therapy. The pharmacosensitivity assay was performed on liver tumor cells with 26 drugs (5 of which were biological response modifiers). Doxorubicin, methotrexate, fluoruracil, floxuridine, BCNU, ara-C, streptozotocin and interferon alpha (IFNα) were found to have good activity against the colon cancer cells. The 5 most active drugs for each patient and interferon alpha were administered once every 4 weeks for 6 weeks by intra-arterial, intra-hepatic catheter, and also by general systemic administration.

7 patients achieved complete remission for 24 or more months, with 4 patients still in complete remission after 4 years. Partial remission was achieved in 7 patients for 7 to 35 months; of these, 4 had complete liver resection and 1 had partial liver resection after the chemotherapy was completed. 6 patients demonstrated no change for 8 months. 1 patient showed disease progression. Minimal side-effects (leukopenia and thrombocytopenia) were observed and were reversible. The experiment showed that the procedure offers an effective choice of drugs for colon cancer with liver metastases with minimal side-effects.

Experiment 33: 86 cancer patients with liver metastases, all of which were previously treated unsuccessfully with conventional therapy, had primary tumors were of the digestive tract (39 patients); genito-urinary tract (10 patients); breast (18 patients); respiratory system (11 patients); and melanoma (8 patients). A catheter was introduced percutaneously to the hepatic artery or implanted during surgery. 30.0 mg of tolazoline hydrochloride was injected intra-arterially to dilate the hepatic artery immediately after placement of the catheter and before chemotherapy. Liver tumor cells demonstrated sensitivity to doxorubicin, mito-C, cisplatin and interferon alpha in the pharmacosensitivity assay and were used for all 86 patients. Two additional drugs (as per patient tumor response) were added to this regimen for each patient. Each patient was treated systemically and intra-arterially for over 3 hours per day, one day every 4 weeks, for 6 courses. Complete remission was achieved in 24 patients with a duration of 8 or more months. 35 patients achieved partial remission for 8–24 months. 24 patients demonstrated NC for 8 months. 3 patients were nonresponsive. Minimal transitory side effects were observed. No local toxicity to the vessels, liver or gallbladder occurred. The experiment showed that the procedure allowed a choice of an effective chemotherapeutic regimen for cancer with liver metastases.

It is understood that the invention is not limited to the particular apparatus and arrangement of steps herein described, but embraces any modified forms that fall within the scope of the following claims.

BIBLIOGRAPHY

Lacombe et al., "Detection of cytarabine resistance in patients with acute myelogenous leukemia using flow cytometry," *Blood*, v84: 716–723 (1994).

Smit et al., "In vitro response of human small-cell lung-cancer cell lines to chemotherapeutic drags: no correlation with clinical data," *International Journal of Cancer*, v51: 72–78 (1992).

Salmon et al. "Primary bioassay of human tumor stem cells," *Science*, v197: 461–463 (1977).

Mossmann, "Rapid calorimetric assay for cellular growth and survival: application to proliferation and cytotoxic assays," *Journal of Immunological Methods*, v65, 55–63 (1983).

Hwang et al., "Prediction of chemotherapy response in human leukemia using in vitro chemosensitivity test," *Leukemia Research*, v17: 685–688 (1993).

Schadendorf et al., "Chemosensitivity testing of human malignant melanoma," *Cancer*, v73, 103–108 (1993).

DeVita, Cancer Principles & Practice of Oncology, J. B. Lippincott Co., 3d ed., 1989.

Arvelo et al., "Response of a multi-drug resistant human small-cell lung cancer xenograft to chemotherapy," *Journal of Cancer Research and Clinical Oncology*, v120: 17–23 (1993).

Van de Loosdrecht et al., "Apoptosis in tumor necrosis factor-alpha-dependent, monocyte-mediated leukemic cell death: a functional, morphologic, and flow-cytometric analysis," *Experimental Hematology*, v21: 1628–1639 (1993).

Darzynkiewicz et al., "Features of apoptotic cells measured by flow cytometry," *Cytometry*, v13: 795–808 (1992).

Christ et al., "Apoptosis induced by oxysterols in murine lymphoma cells and in normal thymocytes," *Immunology*, v78, 455–460 (1993).

Elprana et al., "Chemosensitivity testing of xenografted squamous cell carcinomas of the head and neck region," *Anticancer Research*, v12, 229–2240 (1992).

Camparia et al., "Stroma-supported immunocytometric assay (SIA): a novel method for testing the sensitivity of acute lymphoblastic leukemia cells to cytotoxic drugs," *Leukemia*, v7: 482–488 (1993).

Dao et al., "Natural human interferon-augments apoptosis in activated T-cell line," *Cellular Immunology*, v155: 304–311 (1994).

Thoth et al., "Type I interferon resistance in a colorectal cancer cell line is associated with a more aggressive phenotype in vivo," *British Journal of Cancer*, v65: 365–368 (1992).

Fluckiger et al., "Interleukin 10 induces apoptotic cell death of B-chronic lymphocytic leukemia cells," *Journal of Experimental Medicine*, v179: 91–99 (1994).

Nicoletti et al., "A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry," *Journal of Immunological Methods*, v139: 271–279 (1991).

Bryson, "A flow cytometric study of cell death: failure of some models to correlate with morphological assessment," *Immunology and Cell Biology*, v72, 35–41 (1994).

Huschtscha et al., "Identification of apoptotic and necrotic human leukemic cells by flow cytometry," *Experimental Cell Research*, v212: 161–165 (1994).

Sun et al., "A flow-cytometric method for the separation and quantitation of normal and apoptotic thymocytes," *Analytical Biochemistry*, v204, 351–356 (1992).

What is claimed is:

1. A method for treating non-leukemic cancers in humans comprising:

(a) preparing a cancer cell suspension from a cancer specimen obtained from a human non-leukemic cancer patient;

(b) preparing a control sample from the cancer cell suspension;

(c) preparing several drug samples from several putative cancer cell growth-inhibiting drugs and the cancer cell suspension, each drug sample containing a mixture of the cancer cell suspension and at least one drug; and then

(d) incubating the control sample and drug samples; then

(e) staining the control samples and drug sample with a DNA intercalating dye; then

(f) determining the cancer cell viability in the control sample and the drug samples by use of a flow cytometer and determining the white cell content of the cancer cell suspension and adjusting the determined cancer cell viability of each drug sample and the control sample to compensate for the white cell content of each, whereby an adjusted cancer cell viability of each drug sample and the control sample is obtained; then

(g) comparing the adjusted cancer cell viability of each drug sample with the adjusted cancer cell viability of the control sample; and then

(h) preparing a drug treatment regimen for the human non-leukemic cancer patient containing selected drugs chosen from the several cancer cell growth-inhibiting drugs, the selected drugs corresponding to the drugs in the drug samples having the lowest adjusted cancer cell viability in comparison to the control sample; and then

(i) administering the drug treatment regimen to the human non-leukemic cancer patient in an amount which is effective to inhibit the growth of the cancer.

2. The method of claim 1 wherein step c further comprises calculating the viable cancer cell concentration within the cancer cell suspension, and wherein each drug contained within each drug sample is added in a predetermined aliquot amount proportional to the viable cancer cell concentration.

3. The method of claim 2 wherein each drug sample includes at least one of the following drugs at the following aliquot amounts, and wherein each drug contained within each drug sample is diluted by a pharmaceutical diluent proportionally to a viable cancer cell concentration of about 1×10^6 cells/ml:

amethopterin, 0.25 mg/3.0 ml;
ara-C, 0.60 mg/ml;
BCNU, 0.003 mg/3.0 ml;
bleomycin, 0.05 u/ml;
cis-platin, 0.03 mg/3.0 ml;
cladribine, 0.01 mg/ml;
cyclophosphamide, 0.020 mg/3.0 ml;
dactinomycin, 0.015 mg/ml;
doxorubicin, 0.02 mg/3.0 ml;
DTIC, 0.1 mg/3.0 ml;
etoposide, 0.20 mg/3.0 ml;
fludarabine phosphate, 0.25 mg/3.0 ml;
5-FU, 0.50 mg/3.0 ml;
floxuridine, 0.2 mg/3.0 ml;
hydrea, 0.50 mg/ml;
idamycin, 0.001 mg/3.0 ml;
ifosfamide, 1.5 mg/ml;
levamisole, 0.05 mg/3.0 ml;
mechlorethamine hydrochloride, 0.01 mg/3.0 ml;
medroxyprogesterone, 1.50 mg/3.0 ml;
megestrol acetate, 0.008 mg/3.0 ml;
melphalan, 0.0002 mg/3.0 ml;
mesna, 3.0 mg/3.0 ml;
mito-C, 0.005 mg/ml;
octreotide acetate, 5.0 ug/3.0 ml;
paraplatin, 0.05 mg/ml;
prednisone, 0.6 mg/ml;
retinoic acid, 0.04 mg/3.0 ml;
somatostatin, 5.0 ug/3.0 ml;
streptozocin, 1.0 mg/3.0 ml;
tamoxifen, 0.003 mg/3.0 ml;
taxol, 0.06 mg/3.0 ml;
thio-TEPA, 0.025 mg/ml;
VBL, 0.03 mg/ml; and
VCR, 0.01 mg/ml.

4. The method of claim 2 wherein each drug sample includes at least one of the following drugs at the following aliquot amounts, and wherein each drug contained within each drug sample is diluted by a pharmaceutical diluent proportionally to a viable cancer cell concentration of about 1×10^6 cells/ml:

rIFNα-2a, 30,000 u/ml;
rIL-2, 10 u/ml;
rIFNα-2b, 1,000 neutralizing units/ml;
nHuIFNα, 1×10^3 u/ml;
nHuIFNβ, 1×10^3 u/ml;
nHuIFNπ, 5×10^2 u/ml;
rIFNβ-1b, 1×10^3 u/ml;
rIFNτ-1b, 0.01 μg/ml;
TNF, 1.2×10^2 u/ml;
GM-CSF, 62.5 u/ml; and
G-CSF, 200 u/ml.

5. The method of claim 1 wherein the control sample is a negative control sample, and wherein step (b) further comprises the steps of:

(i) preparing a positive control sample containing the cancer cell suspension and a toxin; then

(ii) incubating the positive control sample; then

(iii) determining the cancer cell viability in the positive control sample and determining the white cell content of the positive control sample and adjusting the determined cancer cell viability of the positive control sample to compensate for the white cell content thereof; and then

(iv) comparing the adjusted cancer cell viability of the positive control sample with the adjusted cancer cell viability of the negative control sample.

6. A method for treating non-leukemic cancers in humans comprising:

(a) preparing a cancer cell suspension from a human non-leukemic cancer patient's cancer specimen; then

(b) calculating the viable cancer cell count within the cancer cell suspension; then

(c) adjusting the volume of the cancer cell suspension to obtain a base cell concentration by diluting the cancer cell suspension with patient medium in proportion with the viable cancer cell count; then

(d) preparing a negative control sample from the cancer cell suspension; and

(e) preparing drug samples, each drug sample containing a mixture of cancer cell suspension, patient medium, and a drug selected from several putative cancer cell growth-inhibiting drugs, wherein each drug sample contains a different drug which is added to the drug sample in an aliquot amount proportional to the base cell concentration; then

(f) incubating the drug samples and negative control sample; then

(g) staining the drug samples and negative control sample with a DNA intercalating dye; then

(h) determining the cancer cell viability in the drug samples and negative control sample by use of a flow cytometer and determining the white cell content of the cancer cell suspension and adjusting the determined cancer cell viability of each drug sample and the control sample to compensate for the white cell content of each, whereby an adjusted cancer cell viability of each drug sample and the control sample is obtained; and then

(i) comparing the adjusted cancer cell viability in the drug samples and negative control sample to determine the pharmacosensitivity of the cancer cells; and then

(j) preparing a drug treatment regimen containing ne or more selected drugs chosen from the several cancer cell growth-inhibiting drugs, the selected drugs corresponding to the drug samples having the lowest adjusted cancer cell viability; and then

(k) administering the drug treatment regimen to the human non-leukemic cancer patient in an amount which is effective to inhibit the growth of the cancer.

7. The method of claim 6 wherein step (d) further comprises

(i) preparing a positive control sample from the cancer cell suspension; then

(ii) incubating the positive control sample; then

(iii) staining the positive control sample with a DNA intercalating dye; and then

(iv.) determining the cancer cell viability in the positive control sample by use of a flow cytometer and determining the white cell content of the positive control sample and adjusting the determined cancer cell viability of the positive control sample to compensate for the white cell content thereof.

8. The method of claim 6 wherein in step (a) the cancer specimen is taken from a non-leukemic metastatic tumor in a human cancer patient suffering from a non-leukemic primary tumor and a non-leukemic metastatic tumor.

9. The method of claim 8 wherein the drug treatment regimen is administered locoregionally.

10. The method of claim 1 wherein in step (a) the cancer specimen is taken from a non-leukemic metastatic tumor in a human cancer patient suffering from a non-leukemic primary tumor and a non-leukemic metasiatic tumor.

11. The method of claim 1 wherein the metastatic tumor is a metastatic tumor of the liver, and further wherein the drug treatment regimen is administered locoregionally via the hepatic artery.

12. The method of claim 1 wherein in step (i) the drug treatment regimen is administered locoregionally.

13. The method of claim 1 wherein in step (i) at least one biological response modifier is administered.

14. The method of claim 1 wherein in step (i) an alpha interferon and a hormone are administered.

* * * * *

Applicants

US006090407A

United States Patent [19]

Knight et al.

[11] Patent Number: 6,090,407

[45] Date of Patent: Jul. 18, 2000

[54] **SMALL PARTICLE LIPOSOME AEROSOLS FOR DELIVERY OF ANTI-CANCER DRUGS**

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[73] Assignee: **Research Development Foundation**, Carson City, Nev.

[21] Appl. No.: **08/933,254**

[22] Filed: **Sep. 23, 1997**

[51] **Int. Cl.**[7] **A61K 9/127**; A61K 9/12

[52] **U.S. Cl.** **424/450**; 424/45; 514/938

[58] **Field of Search** 424/45, 450; 514/938

[56] **References Cited**

U.S. PATENT DOCUMENTS

5,552,156 9/1996 Burke 424/450

5,736,156 4/1998 Burke 424/450

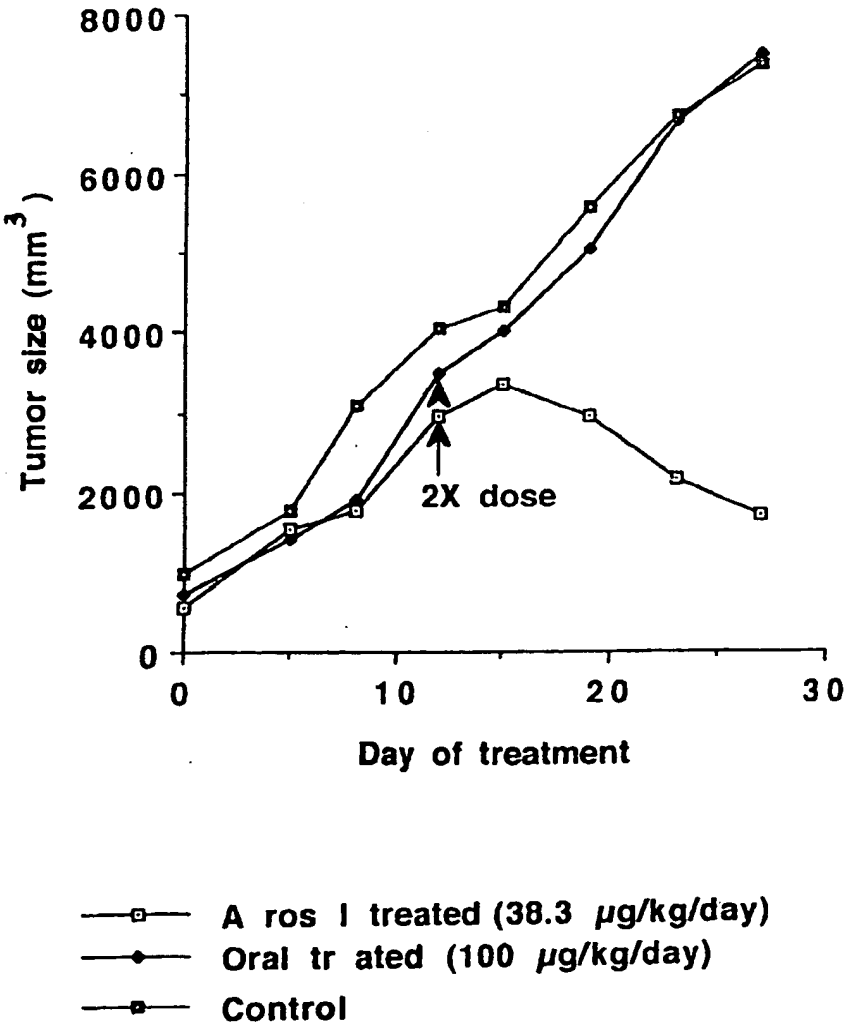
Primary Examiner—Raj Bawa

Attorney, Agent, or Firm—Benjamin Aaron Adler

[57] **ABSTRACT**

The small particle liposome or lipid complex aerosol compounds and methods of treatment of the present invention involve lipid- or water soluble anti-cancer drugs incorporated into liposomes or other lipid complexes. The liposomes and complexes are administered in aqueous dispersions from a jet nebulizer to the respiratory tract of an individual. Various anti-cancer drugs may be used, including 20-S-Camptothecin, 9-Nitro-camptothecin, 9-Amino-camptothecin, 10, 11-methylenedioxy-camptothecin and taxol or its derivatives. Administration of these drugs by inhalation provides faster and more efficient absorption of the anticancer drug than does intramuscular administration or oral administration.

4 Claims, 6 Drawing Sheets



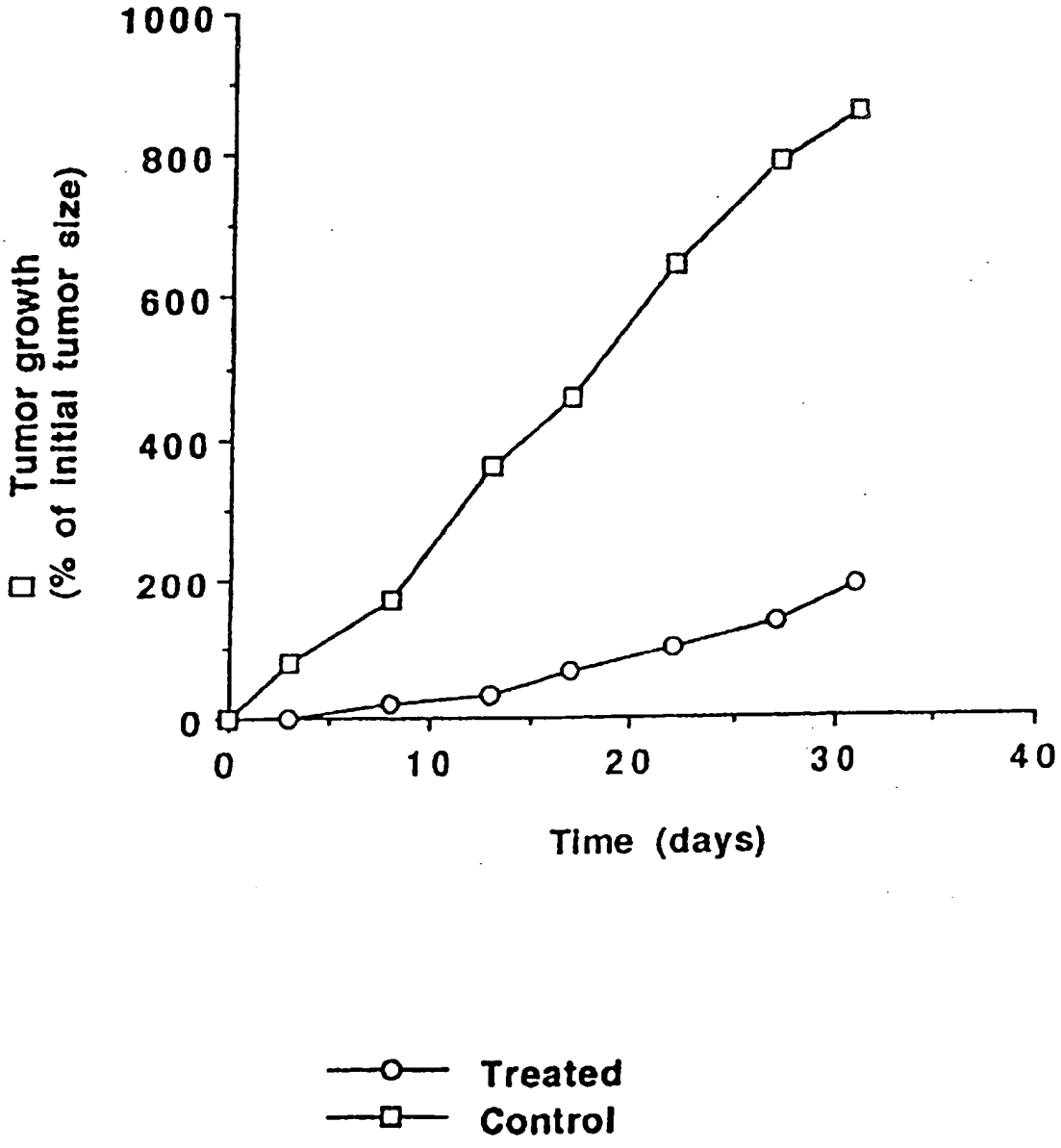

1000
800
600
400
200
0
Tumor growth
(% of initial tumor size)
0
10
20
30
40
Time (days)
Treated
Control

FIG. 1

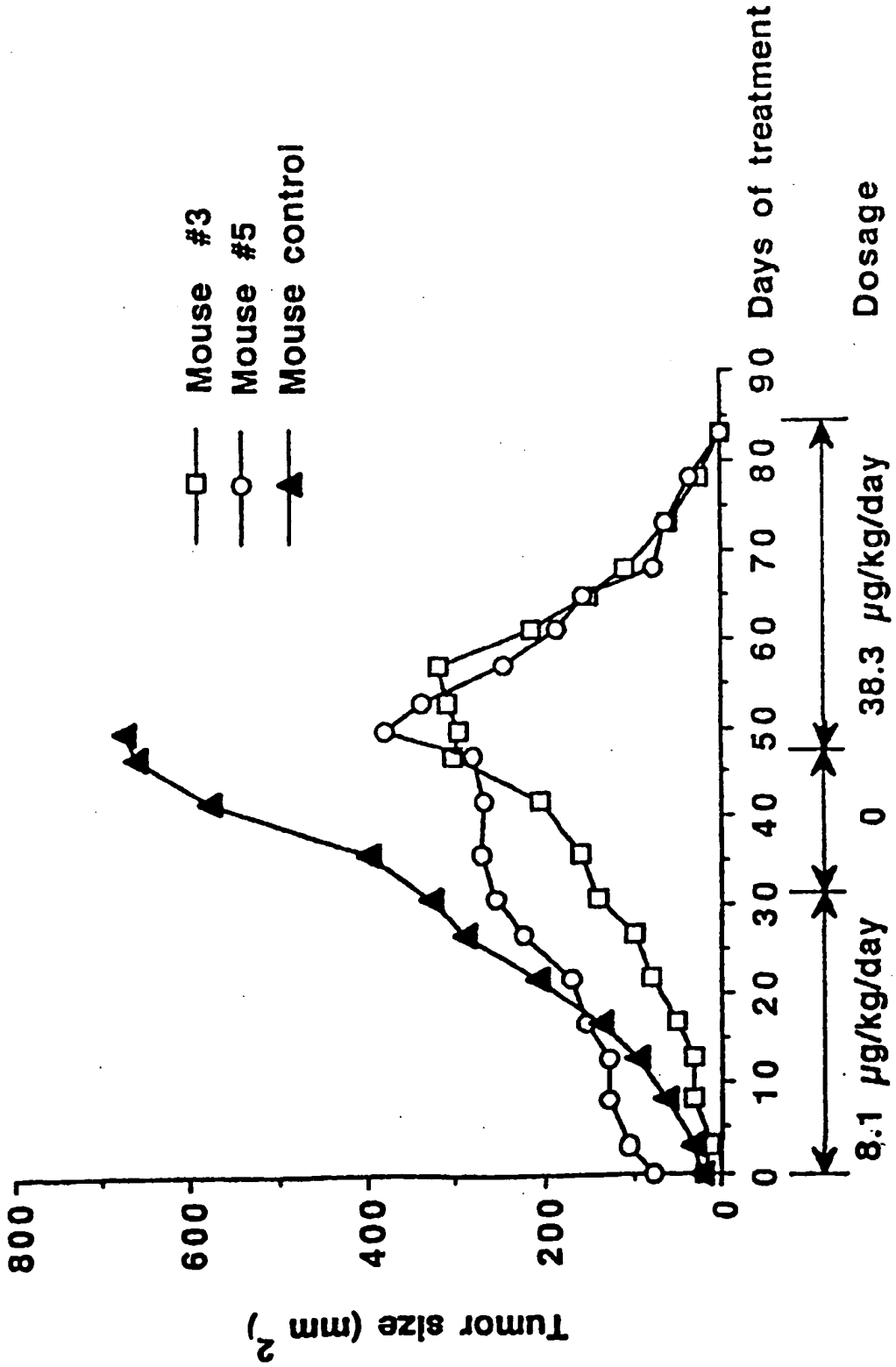

Tumor size (mm 2)
800
600
400
200
0
0 10 20 30 40 50 60 70 80 90 Days of treatment
Dosage
8.1 µg/kg/day
0
38.3 µg/kg/day
Mouse #3
Mouse #5
Mouse control

FIG. 2

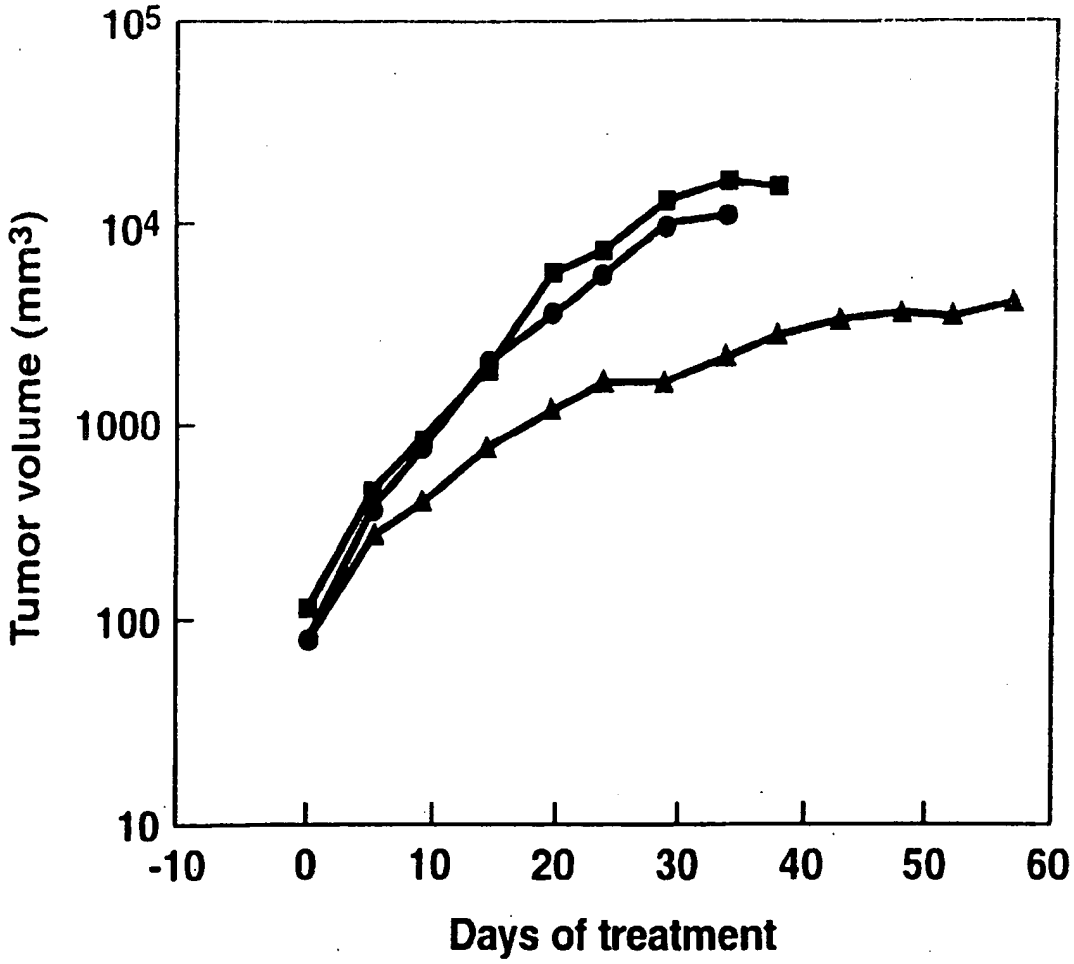

Tumor volume (mm3)
10^5
10^4
1000
100
10
-10
0
10
20
30
40
50
60
Days of treatment

FIG. 3

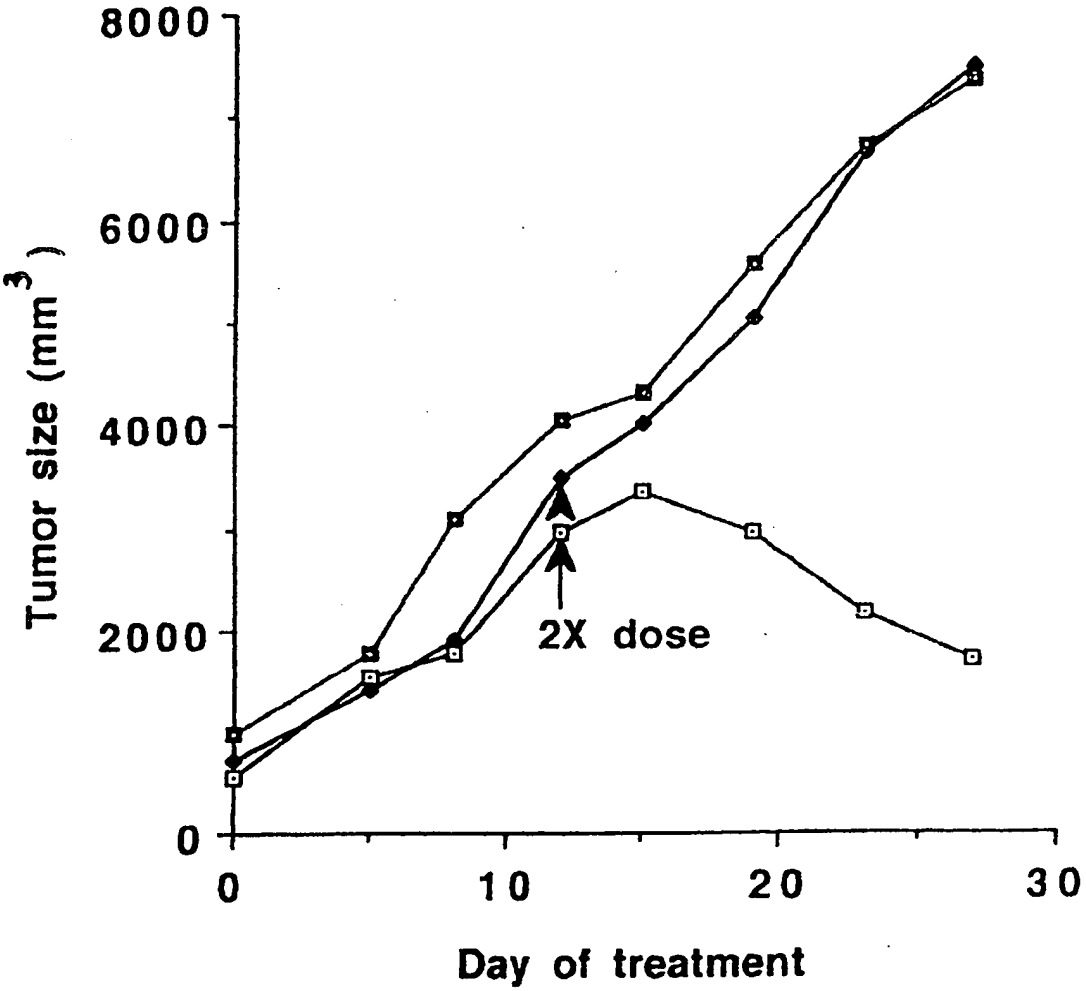

8000
6000
4000
2000
0
Tumor size (mm3)
0
10
20
30
Day of treatment
2X dose
Aerosol treated (38.3 μg/kg/day)
Oral treated (100 μg/kg/day)
Control

FIG. 4

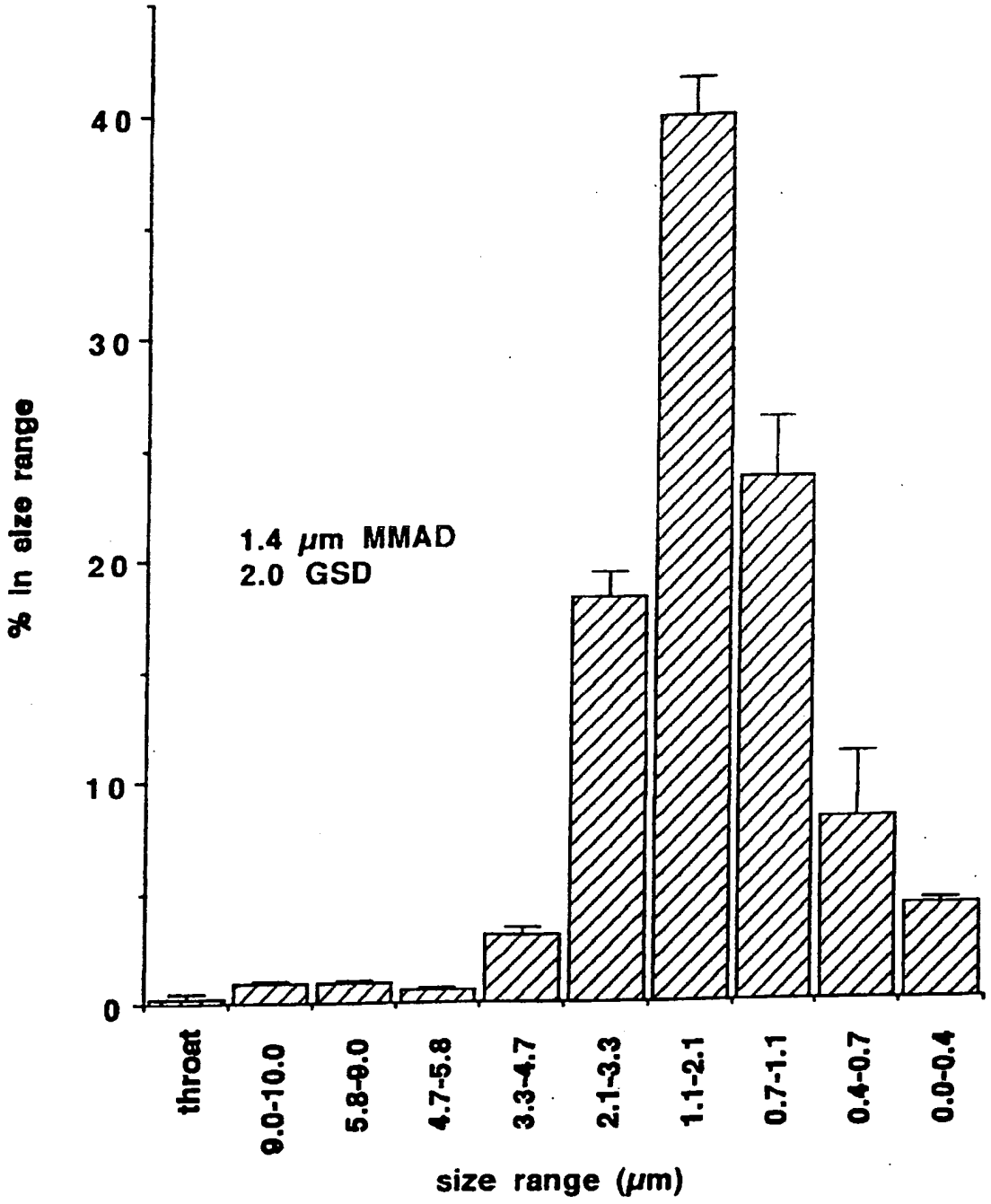

% in size range
40
30
20
10
0
1.4 μm MMAD
2.0 GSD
throat
9.0-10.0
5.8-9.0
4.7-5.8
3.3-4.7
2.1-3.3
1.1-2.1
0.7-1.1
0.4-0.7
0.0-0.4
size range (μm)

FIG. 5

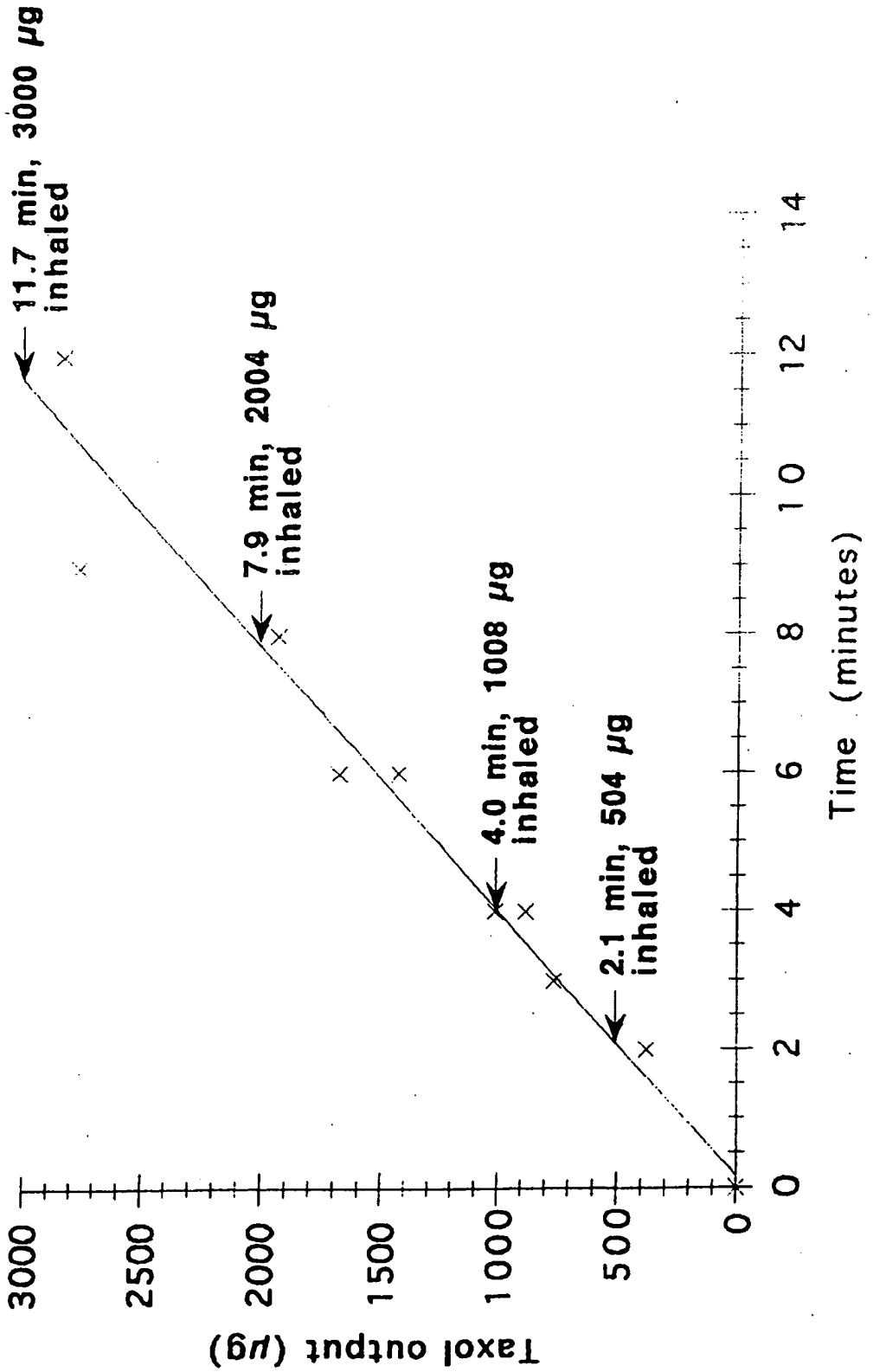

11.7 min, 3000 μg inhaled
7.9 min, 2004 μg inhaled
4.0 min, 1008 μg inhaled
2.1 min, 504 μg inhaled
Taxol output (μg)
3000
2500
2000
1500
1000
500
0
0
2
4
6
8
10
12
14
Time (minutes)

FIG. 6

SMALL PARTICLE LIPOSOME AEROSOLS FOR DELIVERY OF ANTI-CANCER DRUGS

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates generally to the field of pharmacology and cancer treatment. Specifically, the present invention provides formulations and methods for small particle aerosol delivery by inhalation of aqueous dispersions of liposomes carrying anti-cancer drugs to the respiratory tract.

2. Description of the Related Art

Small particle liposome aerosol treatment consists of lipid-soluble or water-soluble anti-cancer drugs incorporated into liposomes, which are administered from aqueous dispersions in a jet nebulizer (see U.S. Pat. No. 5,049,388). Aerosols of 1–3 μm mass median aerodynamic diameter, generated upon nebulization, enable targeted delivery onto surfaces of the respiratory tract. The deposited liposomes subsequently release drug locally within the lung or into the blood circulation with delivery to extra-pulmonary tissue. If the drug is lipid soluble, it will associate with the lipid molecules in a manner specific to the lipid employed, the anti-cancer drug employed and possibly it may be modified further by various soluble constituents which may be included in the suspending aqueous medium. Such soluble constituents may include buffering salts and possibly inositol to enhance the synthesis and secretion of surfactant phospholipid in lung tissue and to minimize respiratory distress already present or that which might result from the aerosol treatment (Hallman, M., et al. Inositol Supplementation in Premature Infants with Respiratory Distress Syndrome, N. Eng. J. Med. 1992 326:1233–1239).

If the drug is water soluble, it may be incorporated by appropriate procedures in aqueous vesicles that exist in concentric spaces between lipid bilayers (lamellae) of the multilamellar liposome. Unilamellar liposomes may be prepared; however, their capacity to entrap either lipid-soluble or water-soluble drugs is diminished since entrapment is restricted to one central vesicle. Additionally, lipid complexes of various sizes can be used.

Nebulization shears liposomes and other lipid complexes to sizes readily discharged from the nozzle of the nebulizer. Liposomes and other lipid complexes up to several microns in diameter are typically sheared to diameters of less than 500 nm, and may be considerably smaller than that depending on the operating characteristics of the nebulizer and other variables. Shearing of water-soluble drugs contained in liposomes or complexes will release appreciable amounts of the water soluble compound, perhaps 50 percent. This is not a contraindication to their use, but it means that two forms of the drug preparation is administered, and the effect includes the therapeutic effect that would be produced by both forms if either form had been given alone. Many other details of liposome aerosol treatment are described in U.S. Pat. No. 5,049,388. Moreover, it is also possible to incorporate more than one drug in a aerosol liposome treatment, either by mixing different drug-containing liposomes, or by using liposomes wherein the drugs have been combined and incorporated together into liposomes.

The prior art is deficient in formulations and methods for small particle aerosol delivery of aqueous dispersions of liposomes or lipid complexes containing anti-cancer drugs. The present invention fulfills this long-standing need and desire in the art.

SUMMARY OF THE INVENTION

The small particle liposome or lipid complex aerosol compounds and methods of treatment of the present invention involve lipid-soluble or water-soluble anti-cancer drugs incorporated into liposomes or other lipid complexes. These drug-carrying lipids then are administered in aqueous dispersions from a jet nebulizer. The present invention demonstrates that speedier and more efficient systemic absorption of drug is actualized after pulmonary administration by aerosol than is actualized by intramuscular or oral administration.

One object of the present invention is to provide a method for treating cancer, comprising the step of delivering, via small particle aerosol, aqueous dispersions of anti-cancer drugs to the respiratory tract of an individual in need of such treatment. Examples of anticancer drugs available for use in this embodiment of the invention include, but are not limited to, 20-S-camptothecin, 9-nitro-camptothecin, 9-amino-camptothecin, 10, 11 -methylenedioxy-camptothecin, taxol, taxol-A, mitotane, methotrexate, mercaptopurine, lomustine, interferon, 5-fluorouracil and etopiside. In a more preferred embodiment of this object, the anti-cancer drug is selected from the group consisting of 20-S-camptothecin, 9-nitro-camptothecin, 9-amino-camptothecin, 10, 11-methylenedioxy-camptothecin and taxol. Additionally, in a preferred embodiment of the present objective, the delivery of the anticancer drug is performed by a jet nebulizer.

In another object of the present invention, there is provided a lipid complex or liposome for delivery of anticancer drugs via small particle aerosols comprising an anticancer drug and a lipid, wherein the anticancer drug is at a concentration not exceeding about 10% of the total volume of the preparation and a ratio of the anticancer drug to the suitable solvent is in the range of about 1:1 to about 1:200, preferably in a range of about 1:10 to about 1:100, and most preferably in a range of about 1:10 to about 1:50 (wt:wt) of the preparation. One specific embodiment of this object includes 9-nitro-camptothecin and dilauroylphosphatidylcholine in a ratio of about 1:10 to 1:50 wt:wt; with a particularly preferred embodiment having a 9-nitro-camptothecin and dilauroylphosphatidylcholine of about 1:50 wt:wt. In another embodiment, there is provided a liposome for delivery of anticancer drugs via small particle aerosols comprising Taxol and dilauroylphosphatidylcholine in a ratio of about 1:30 wt:wt.

In yet another embodiment of the present invention, there is provided a liposome produced by the following steps: dissolving a lipid-soluble anticancer drug in a solvent suitable for dissolving the anticancer drug to produce dissolved anticancer drug; adding the dissolved anticancer drug to a dissolved lipid suitable for formulation and delivery of drugs by aerosol to produce a solution, wherein the dissolved anticancer drug is at a concentration not exceeding about 10% of the total volume of the solution and a ratio of the anticancer drug to the lipid is in the range of about 1:1 to about 1:200, preferably in a range of about 1:10 to about 1:100, and most preferably in a range of about 1:10 to about 1:50 (wt:wt) of the solution; and freezing and lyophilizing the solution. At this point, the solution may be stored frozen for later use or dissolved in sterile water for use, producing a suspension, wherein the concentration of the anticancer drug in the sterile water in the suspension is no more than about 5.0 mg/ml.

A preferred embodiment of the above object provides liposomal preparations of 20-S-camptothecin (CPT), 9-nitrocamptothecin (9-NC) and other lipid soluble camptothecin derivatives, produced by the following steps: preparing concentrated stock solutions of said 20-S-camptothecin (CPT), 9-nitrocamptothecin (9-NC) or other-lipid soluble camptothecin derivatives and lipids in

compatible solvents; adding appropriate volumes of the 20-S-camptothecin (CPT), 9-nitrocamptothecin (9-NC) or other-lipid soluble camptothecin derivative and lipid concentrated stock solutions to a volume of t-butanol to form a second solution, wherein a concentration of said 20-S-camptothecin (CPT), 9-nitrocamptothecin (9-NC) and other lipid soluble camptothecin derivatives does not exceed 10% of said second solution and wherein a ratio of drug to lipid is in the range of about 1:1 to about 1:200, preferably in a range of about 1:10 to about 1:100, and most preferably in a range of about 1:10 to about 1:50 (wt:wt) in said second solution; freezing said second solution; and lyopbilizing said second solution to produce a powder preparation. At this point, the powder preparation may be stored frozen for later use or dissolved in sterile water producing a suspension, wherein a concentration of said anticancer drug in said suspension is no more than about 5 mg/ml.

A more particular embodiment provides liposomes produced by the following steps: preparing a concentrated stock solutions of anticancer drug, for example 100 mg CPT in 1 ml t-butanol or 100 mg 9-NC in DMSO, preparing a stock solution of lipid, for example, 100 mg DLPC in 1 ml butanol; adding appropriate volumes of said concentrated stock solutions to a volume of t-butanol to form a second solution wherein a final volume is about 10 ml, a volume of DMSO, if any, does not exceed 10% (vol:vol) of said final volume, a concentration of anticancer drug does not exceed 10% (wt:wt) of the total volume, and wherein a ratio of drug to lipid is in a range of about 1:1 to about 1:200, preferably in a range of about 1:10 to about 1:100, and most preferably in a range of about 1:10 to about 1:50 (wt:wt); freezing said second solution; and lyophilizing said frozen solution to produce a powder preparation. Tthe powder preparation may then be stored frozen for later use or dissolved in sterile water producing a suspension. Generally, the concentration of the anticancer drug in the suspension is no more than about 5 mg/ml.

Another preferred embodiment of the object above provides a liposome produced by the following steps: mixing taxol with synthetic alpha lecithin: dilauroylphosphatidylcholine; dissolving the taxol-DLPC in t-butanol to produce a preparation; and freezing and lyophilizing the preparation. Liposomes are produced by adding sterile, pure water at a temperature above 25° C., wherein the final concentration of taxol to dilauroylphosphatidylcholine is about 1:1 to about 1:200, preferably in a range of about 1:10 to about 1:100, and most preferably in a range of about 1:25 to about 1:40 (wt:wt). In addition to alpha lecithin, other natural or synthetic lecithins may be used, including but not limited to egg yolk phosphatidylcholine, hydrogenated soybean phosphatidylcholine, dimyristophosphatidylcholine, diolyeolyl-dipalmitoyleolylphosphatidylcholine and dipalmitoyl phosphatidylcholine.

The efficiency of incorporation of 9-NC and other camptothecin derivatives and anticancer drugs into liposomes can be tested by layering an aqueous dispersion of lyophilized drug-liposome preparation over a Percoll™ gradient and centrifuging. Unincorporated drug collects at the bottom of the tube, but drug incorporated into liposomes collects at the interface between the Percoll gradient and the water phase. One qualitative test of incorporation efficiency is the observation of drug crystals when the dispersion of drug-liposomes are examined by microscopy under polarized light. Other methods are also available, for example, analytical HPLC methods can be used to quantitatively assess non-encapsulated, crystalized drug.

Other and further aspects, features, and advantages of the present invention will be apparent from the following description of the presently preferred embodiments of the invention. These embodiments are given for the purpose of disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

The appended drawings have been included herein so that the above-recited features, advantages and objects of the invention will become clear and can be understood in detail. These drawings form a part of the specification. It is to be noted, however, that the appended drawings illustrate preferred embodiments of the invention and should not be considered to limit the scope of the invention.

FIG. 1: shows the effect of treatment with 9-NC-DLPC liposome aerosol on xenografted human breast cancer in nude mice.

FIG. 2: shows the effect of further treatment with 9-NC-DLPC liposome aerosol in mice selected from FIG. 1.

FIG. 3: shows the effect of treatment with 9-NC-DLPC liposome aerosol on xenografted human colon cancer (Squires) in nude mice.

FIG. 4: shows the effect of treatment with 9-NC-DLPC by liposome aerosol or by oral administration on the growth of human lung cancer xenografts (Spark) in nude mice as measured by tumor volume.

FIG. 5: shows the output of Taxol-DLPC liposomes (1:30; 1 mg/ml) with the Aerotech II nebulizer.

FIG. 6: shows the recovery of taxol from the preparation described in FIG. 5; taxol-DLPC dosage at 15 bpm, 500 ml TV.

DETAILED DESCRIPTION OF THE INVENTION

The following definitions are provided. Terms not specifically defined are meant to interpreted as is customary in the art.

As used herein, the term "aerosols" refers to dispersions in air of solid or liquid particles, of fine enough particle size and consequent low settling velocities to have relative airborne stability (See Knight, V., Viral and Mycoplasmal Infections of the Respiratory Tract. 1973, Lea and Febiger, Phila. Pa., pp. 2). "Liposome aerosols" consist of aqueous droplets within which are dispersed one or more particles of liposomes or liposomes containing one or more medications intended for delivery to the respiratory tract of man or animals (Knight, V. and Waldrep, J. C. Liposome Aerosol for Delivery of Asthma Medications; see also In Kay, B., Allergy and Allergic Diseases, 1997, Blackwell Publications, Oxford, England, Vol. I pp. 730–741). The size of the aerosol droplets defined for this application are those described in U.S. Pat. No. 5,049,338, namely mass median aerodynamic diameter (MMAD) of 1–3 μm with a geometric standard deviation of about 1.8–2.2. However, with low concentrations of 9-NC and possibly other camptothecin derivatives, the MMAD may be less than 1 μm, such as 0.8 μm. Based on the studies disclosed by the present invention, the liposomes may constitute substantially all of the volume of the droplet when it has equilibrated to ambient relative humidity.

As used herein, the "Weibel Lung Model" refers to a classification of the structure of the human lungs that recognizes 23 consecutive branchings of the airways of humans. The trachea is labeled 0, bronchi and bronchioles extend through branches 16. These portions of the airways contain ciliated epithelium and mucus glands. Together they constitute the mucociliary blanket. Branchings 17–23 com-

pose the alveolar portion of the lung and do not have a mucociliary blanket. Thus, particles deposited here are not carried up the airway to be swallowed.

As used herein, the terms "20-S-camptothecin" or "CPT" refers to a plant alkaloid with anti-cancer properties.

As used herein, the terms "9-nitro-camptothecin" or "9-NC", "9-amino-camptothecin" or "9-AC," and "10, 11 -methylenedioxy-camptothecin" or "MDC" refer to active anti-cancer drugs derived from 20-S-camptothecin that are insoluble in water but are soluble in certain lipid solvents.

As used herein, the terms "dilauroylphosphatidylcholine" or "DLPC" is a lipid used to formulate liposomes.

The present invention is directed to a method for treating cancer, comprising the step of delivering, via small particle aerosol, aqueous dispersions of anti-cancer drugs to the respiratory tract of an individual in need of such treatment. In a preferred embodiment of this object, the anti-cancer drug is selected from the group consisting of 20-S-camptothecin, 9-nitro-camptothecin, 9-amino-camptothecin and 10, 11-methylenedioxy-camptothecin. Additionally, in a preferred embodiment of the present objective, the delivery of the anticancer drug is performed by a jet nebulizer.

Additionally, there is a liposome for delivery of anticancer drugs via small particle aerosols comprising an anticancer drug and a lipid, wherein the anticancer drug is at a concentration not exceeding about 10% of the total volume of the preparation and a ratio of the anticancer drug to the suitable solvent is in the range of about 1:1 to about 1:200, preferably in a range of about 1:10 to about 1:100, and most preferably in a range of about 1:10 to about 1:50 (wt:wt) of the preparation. One specific embodiment of this object includes 9-nitro-camptothecin and dilauroylphosphatidylcholine in a ratio of about 1:10 to 1:50 wt:wt; with a particularly preferred embodiment having a 9-nitro-camptothecin and dilauroylphosphatidylcholine of about 1:50 wt:wt.

Taxol is another anti-cancer drug that is lipid soluble and is incorporated easily into a liposome formulation. The optimal ratio for taxol and dilauroylphosphatidylcholine is a ratio of about 1:1 to about 1:200, preferably in a range of about 1:10 to about 1:100, and more preferably in a range of about 1:25 to about 1:40 (wt:wt). A most preferred embodiment provides a taxol to DLPC ratio of about 1:30. Taxol is directly dissolved in t-butanol without use of DMSO as is used for some camptothecins. The Taxol liposomal preparation is otherwise similar to that of the camptothecins.

Further, the present invention is directed to liposomes for delivery of anticancer drugs via small particle aerosols produced by the following steps: dissolving a lipid-soluble anticancer drug in a solvent suitable for dissolving the anticancer drug to produce dissolved anticancer drug; adding the dissolved anticancer drug to a dissolved lipid suitable for formulation and delivery of drugs by aerosol to produce a solution, wherein the dissolved anticancer drug is at a concentration not exceeding about 10% of the total volume of the solution and a ratio of the anticancer drug to the suitable solvent is in the range of about 1:1 to about 1:200 of the solution; and freezing and lyophilizing the solution. At this point, the solution may be stored frozen for later use or dissolved in sterile water to produce a suspension, wherein the concentration of the anticancer drug in the sterile water in the suspension is no more than about 5.0 mg/ml. A particular embodiment of the present invention provides a liposome produced by the following steps: dissolving a lipid-soluble anticancer drug selected from the group of 20-S-camptothecin, 9-nitro-camptothecin, 9-amino-camptothecin and 10, 1 1-methylenedioxy-camptothecin in 100% DMSO to produce dissolved anticancer drug; and adding said dissolved anticancer drug to dilauroylphosphatidylcholine dissolved in t-butanol to produce a solution, wherein the dissolved anticancer drug is at a concentration not exceeding about 5% of the total volume of the solution and the ratio of anticancer drug to dilauroylphosphatidylcholine is about 1:50 in the solution. The solution is frozen and lyophilized overnight. For use, the lyophilized solution is suspended in appropriate volumes of sterile, distilled water. In addition, other methods of liposome preparation known in the art may be utilized, for example, rotary evaporation can be used instead of lyophilization.

9-NC-DLPC aerosol is prepared by first dissolving the drug in DMSO; to do so, heating to 50–60° C. may be required. This solution is added to a larger volume of t-butanol, such that the DMSO solution does not exceed 5–10% of the total t-butanol and DMSO volume combined. The organic solvents DMSO and t-butanol are evaporated from the solution on liquid nitrogen resulting in a slightly yellow powder. For use, distilled sterile water is added to the vials containing the drug at the appropriate concentration and added to the reservoir of the nebulizer. The Aerotech II™ nebulizer CIS-USA, Inc., Bedford Mass. is currently employed, but other nebulizers with similar aerosol-generating properties may be used.

A particular embodiment of the present invention is directed to a liposome produced by the following steps: mixing taxol with synthetic alpha lecithin: dilauroylphosphatidylcholine; dissolving the taxol-DLPC in t-butanol to produce a solution; and freezing and lyophilizing the solution. Liposomes are produced by adding sterile, pure water at a temperature above 25° C., wherein the final concentration of taxol to dilauroylphosphatidylcholine is about 1:1 to about 1:200, preferably in a range of about 1:10 to about 1:100, and more preferably in a range of about 1:25 to about 1:40 (wt:wt).

It is contemplated specifically that the pharmaceutical compositions of the present invention be used for aerosol delivery of aqueous dispersions of liposomes carrying anticancer drugs to the respiratory tract. A person having ordinary skill in this art would readily be able to determine, without undue experimentation, the appropriate dosages of these aerosol formulations. When used in vivo for therapy, the aerosol formulations of the present invention are administered to the patient in therapeutically effective amounts; i.e., amounts that eliminate or reduce the tumor burden. As with all pharmaceuticals, the dose and dosage regimen will depend upon the nature of the cancer (primary or metastatic), the characteristics of the particular drug (e.g., its therapeutic index), the patient, the patient's history and other factors. The amount of aerosol formulation administered will typically be in the range of about 8 μg/kg of patient weight/day to about 100 μg/kg of patient weight/day for 9-NC. Again, dose and dosage regimen will vary depending on a number of factors known to those skilled in the art. See Remington's Pharmaceutical Science, 17th Ed. (1990) Mark Publishing Co., Easton, Pa.; and *Goodman and Gilman's: The Pharmacological Basis of Therapeutics* 8th Ed (1990) Pergamon Press.

The small particle liposome aerosol compounds and methods of treatment of the present invention involve lipid- or water-soluble anti-cancer drugs incorporated into liposomes. The liposomes are administered in aqueous dispersions from a jet nebulizer. Various anti-cancer drugs may be used, including 20-S-camptothecin, 9-nitro-camptothecin, 9-amino-camptothecin, 10, 11 -methylenedioxy-camptothecin and taxol.

The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion:

EXAMPLE 1

Anti-cancer drugs CPT and its 9-NC derivative

Representative of the anti-cancer drugs of the present invention are 20-S-camptothecin and its 9-NC derivative. Other camptothecins such as 9-AC and 10, 11-MDC, as well as other anticancer formulations known in the art, also possess properties suitable for use. All these anti-cancer drugs may be used in liposome formulations.

Table 1 shows a comparison of blood or plasma concentrations of 9-NC and the time of peak concentrations following oral administration to humans, dogs and mice. (Hinz, H. R, et. al. Pharmacokinetics of the in vivo and in vitro conversion of 9-Nitro 20-(S)-camptothecin to 9-Amino-20-(S)-camptothecin in Humans, Dogs and Mice. *Cancer Research* 1994 54:3096–3100). Single oral doses ranged from 0.1 to 1.0 mg/kg for humans and dogs and were 4.1 mg/kg for mice. There may be some differences in pharmacokinetics between CPT and 9-NC, but it is a reasonable possibility that the foregoing differences are predictive of the properties of both agents.

TABLE 1

Comparison of 9-NC concentrations in blood or plasma following single oral, intranasal or aerosol dosage

		Blood or plasma concentration	Time of maximum concentration
	Oral dose (Single doses)		
Human	0.1 mg/kg (7 mg/dose)	483 ng/ml	3.4 h (T½ = 2.5 h)
	1.0 mg/kg (70 mg/dose)	1247 ng/ml	5.3 h (T½ = 4.9 h)
Dog	1.0 mg/kg (10 mg/dose)	19.1 ng/ml	0.7 h (T½ = 6.4 h)
Mouse	4.1 mg/kg (0.124 mg/dose) *Intranasal	732 ng/ml	6 min (T½ = 10 h)
Mouse	233 μg/kg (7 μg/mouse) *Inhaled in liposome aerosol (30 min)	213 ng/ml	end of instillation
Mouse	16.2 μg/kg (486 ng/mouse)	13.9 ng/ml	end of aerosolization

*Intranasal and aerosol results from Baylor Aerosol Laboratory, the others from Hinz, H. R. (see text for reference)

Table 2 describes the method of calculating aerosol dosage in the mouse, and this is the basis for determining dosages cited in Table 1. Much of the inhaled drug is deposited in the nasopharynx of the mouse because of the complex nose structure of rodents. Similarly, nasal instillation leads to high nasopharyngeal deposition. Material deposited here is promptly transported to the esophageal orifice and swallowed. About 10–15 percent of the inhaled aerosol dose, however, will penetrate to the peripheral lungs.

In humans with mouth breathing, only small amounts of the aerosol particles will deposit in the mouth and virtually none will deposit in the nasopharynx. Material deposited in the central airways is returned to the pharynx by mucociliary action where it is swallowed. In the case of primary lung tumors which often occur at bronchial bifurcations, drug will deposit on tumor surfaces and be adsorbed directly into the tumor mass. Material deposited beyond the 16th Weibel generation, which is beyond the ciliated epithelium, will not be moved upward; thus, in tumors within the peripheral lung parenchyma, the drug will deposit in adjacent areas and be absorbed directly into the tumor mass. In addition, a major advantage of the present invention is that drug deposited beyond the ciliated epithelium is picked up by capillaries in the interstitial space and the lymphatics of the lungs and will enter the circulation.

TABLE 2

Calculation of deposited doses of 9-NC in different species when administered by aerosol
NEBULIZER: AeroTech II; 10 L/min; Measured 1.7% efficiency
RESERVOIR: 9-Nitrocamptothecin (9NC)-DLPC liposomes: 100, 500 or 1,000 μg 9NC/mL and 1:50 (w/w) mg DLPC/mL
Dose Calculation

Species	Drug	MMAD	9NC in Resv (μg/mL)	9NC in Aerosol (μg/L)	Body Wt (kg)	Rx Time (min/day)	K*	Drug Dose (μg/kg/min)	Total Drug (μg/kg/day)	(μg/day)	Resp Secr (μg/mL)**	Weibel Generations: 0–16	Weibel Generations: 17–23 (μg/mL)***
Human [adult]	9NC	0.8	100	1.8	70	15	0.076	0.14	2.0	142	0.68	1.4	5.8
Human [adult]	9NC	0.8	100	1.8	70	30	0.076	0.14	4.1	284	1.35	2.7	11.7
Human [adult]	9NC	1.2	500	8.5	70	15	0.076	0.64	9.6	675	3.21	6.5	27.7

TABLE 2-continued

Calculation of deposited doses of 9-NC in different species when administered by aerosol
NEBULIZER: AeroTech II; 10 L/min; Measured 1.7% efficiency
RESERVOIR: 9-Nitrocamptothecin (9NC)-DLPC liposomes: 100, 500 or 1,000 μg 9NC/mL and 1:50 (w/w) mg DLPC/ml.
Dose Calculation

Species	Drug	MMAD	9NC in Resv (μg/mL)	9NC in Aerosol (μg/L)	Body Wt (kg)	Rx Time (min/day)	K*	Drug Dose (μg/kg/min)	Total Drug (μg/kg/day)	(μg/day)	Resp Secr (μg/mL)**	Weibel Generations: 0–16 (μg/mL)***	17–23 (μg/mL)***
Human [adult]	9NC	1.2	500	8.5	70	30	0.076	0.64	19.3	1,353	6.44	13.0	55.5
Human [adult]	9NC	1.5	1,000	15.9	70	15	0.076	1.20	18.0	1,259	6.00	12.1	51.6
Human [adult]	9NC	1.5	1,000	15.9	70	30	0.076	1.20	36.0	2,518	11.99	24.2	103.1
Dog	9NC	1.2	500	8.5	30	15	0.100	0.85	12.8	383	4.26	8.6	36.7
Dog	9NC	1.2	500	8.5	30	30	0.100	0.85	25.6	767	8.52	17.2	73.4
Cotton Rat	9NC	1.2	500	8.5	0.075	30	0.350	2.98	89.5	6.71	29.8		
Cotton Rat	9NC	1.2	500	8.5	0.075	60	0.350	2.98	178.9	13.42	59.6		
Cotton Rat	9NC	1.2	500	8.5	0.068	30	0.350	2.98	89.5	6.08	29.8		
Mouse	9NC	0.8	100	1.8	0.030	15	0.300	0.54	8.1	0.24	2.7		
Mouse	9NC	0.8	100	1.8	0.030	30	0.300	0.54	16.1	0.48	5.4		
Mouse	9NC	1.2	500	8.5	0.030	15	0.300	2.55	38.3	1.15	12.8		
Mouse	9NC	1.2	500	8.5	0.030	30	0.300	2.56	76.7	2.30	25.6		
Mouse	9NC	1.2	500	8.5	0.030	60	0.300	2.56	153.4	4.60	51.1		
Mouse	9NC	1.2	500	8.5	0.030	120	0.300	2.56	306.7	9.20	102.2		
Mouse	9NC	1.5	1,000	15.9	0.030	15	0.300	4.76	71.4	2.14	23.8		
Mouse	9NC	1.5	1,000	15.9	0.030	30	0.300	4.77	143.1	4.29	47.7		

*K(human/adult): 0.108 L-min/kg × 0.7; Assuming Nose and mouth breathing, Mouth-only = ½
K(cotton rat): 0.7 L-min/kg × 0.5
K(mouse): 1 L-min/kg × 0.3
K(dog, golden): 0.2 L-min/kg × 0.5
**Estimated Peak after each treatment: secretion volume = 1 mL/kg
***Based on data from Patton (3570) and Philadelphia info; Mouth-only breathing in man

Table 3 shows the tissue distribution of CPT following 15 minutes of nebulization in DLPC liposome aerosol. The deposited dose was calculated to be 486 ng per mouse. The mean concentrations in lungs and liver were similar with smaller concentrations in the other sites examined. Table 4 shows tissue distributions over a period of one hour following intranasal instillation of 7 μg per mouse (233 μg/kg). Drug was cleared promptly from the lungs so that by 15 minutes after stopping nebulization only negligible amounts of drug were present in the lungs. Liver, kidney and spleen had substantial amounts of drug initially which gradually diminished through the one hour of study. Interestingly, blood concentrations were the least throughout the study. These studies indicate substantial immediate deposition of drug in the lungs with rapid clearance to the viscera. The amount of drug contributed by absorption from swallowed drug is uncertain.

TABLE 3

Tissue distribution of CPT following 15 minutes inhalation of CPT liposome aerosol

Animal	Organ	CPT (ng/gm) tissue
1	Lung	52.0
	Liver	44.3
	Spleen	12.0
	Kidney	29.3
	Blood	7.1
2	Lung	48.0
	Liver	44.3
	Spleen	16.4
	Kidney	21.8
	Blood	8.3
3	Lung	27.0

TABLE 3-continued

Tissue distribution of CPT following 15 minutes inhalation of CPT liposome aerosol

Animal	Organ	
	Liver	21.9
	Spleen	11.4
	Kidney	18.0
	Blood	22.6
4	Lung	77.5
	Liver	178.0
	Spleen	25.0
	Kidney	50.0
	Blood	17.7
		MEAN (±SD) CPT (ng/gm tissue)
	Lung	51.1 ± 20.7
	Liver	72.1 ± 71.4
	Spleen	16.2 ± 6.3
	Kidney	29.8 ± 14.3
	Blood	13.9 ± 7.5

The CPT concentration in the liposomal preparation in the nebulizer was 0.2 ng/ml aerosol was generated with an Aerotech II nebulizer operating at a flow rate of 10 L/min.

TABLE 4

Time dependent organ distribution of CPT after intranasal administration

Organ	Time (minutes) 0	15	30	60
	ng/gm of tissue			
Lung	1287 ± 657	19 ± 3	36 ± 23	7 ± 3
Liver	651 ± 418	255 ± 101	66 ± 17	34 ± 7
Kidney	542 ± 174	190 ± 57	49 ± 13	24 ± 21

TABLE 4-continued

Time dependent organ distribution of CPT after intranasal administration

Organ	Time (minutes) 0	15	30	60
	ng/gm of tissue			
Spleen	351 ± 137	84 ± 32	21 ± 8	7 ± 2
Blood	213 ± 19	53 ± 20	8 ± 3	4 ± 2

Remarks:
CPT was administered in liposomal formulation prepared with DLPC with initial drug concentration 0.2 mg/ml.
35 μl. of suspension was installed to each animal (group of 3 animals was treated for each time point).

Table 5 shows the distribution of drug in blood and viscera following intramuscular injection of CPT. Drug disappeared very slowly from the site of intramuscular injection in the first 12 hours, with only very small concentrations detected in the liver and virtually no drug present at other sites. Concentrations in the blood were negligible throughout the study. The dose administered was the same as that given by intranasal instillation. These findings indicate a speedier and more efficient systemic absorption of drug after pulmonary administration of drug than by the intramuscular route. It is likely that deposition in organs and vascular spaces will increase the opportunity for exposure to albumin molecules and degradation to the carboxyl form of the drug.

TABLE 5

Time dependent organ distribution of CPT after intramuscular administration

Organ	Time (minutes) 0	30	60	1200
	ng/gm tissue			
Lung	2 ± 1	4 ± 2	3 ± 3	4 ± 3
Liver	3 ± 1	87 ± 74	136 ± 107	126 ± 116
Spleen	2 ± 1	18 ± 9	11 ± 5	5 ± 1
Kidney	2 ± 0	40 ± 14	26 ± 7	15 ± 5
Blood	2 ± 1	12 ± 5	8 ± 1	4 ± 1
Site of inj.	6918 ± 265	4309 ± 1548	4609 ± 1412	1544 ± 751

Remarks
CPT initial stock 5 mg/ml in DMSO was suspended in saline (1.4 μl. stock + 48.6 μL saline) and total 50 μL of suspension was injected i.m. in each mice.
Group of 3 animals was treated for each time point.

EXAMPLE 2

Stability of Liposomes Consisting of DLPC and 9-AC

Table 6 shows the stability of liposomes with fixed weight ratio of 9-NC and DLPC of 1:50 (w/w) but with increasing concentrations of constituents from 0.1 mg/ml to 1.0 mg/ml of drug. The samples were tested under various conditions after vortexing, but before start of nebulization, after nebulization for 1.5 to 2 minutes (sample taken from the fluid in the reservoir of the nebulizer) and from the aerosol that was collected in an all-glass impinger (All-Glass Impinger, Ace Glass Co., Vineland N.J.).

TABLE 6

Liposome particle size and drug crystal formation in preparations of 9-NC DLPC liposome formulations

Concentration (mg/ml) 9NC	DLPC	Sample	Liposome particle size, nm	Crystals presence (visual estimation)
0.1	5.0	1	8006	-
		2	798	+
		3	352	-
0.2	10.0	1	6201	-
		2	434	+
		3	812	-
1.0	50.0	1	5448	++
		2	718	++
		3	816	+

1 sample was tested after vortexing
2 samples was taken from nebulizer reservoir after 1.5 to 2 minutes of aerosolization
3 sample was from aerosol collected in an AGI containing water

The most stable preparation was the one with lowest concentration of constituents. A few crystals appeared in the reservoir following nebulization. Nebulization caused a ten-fold reduction in the diameter of the liposome particles, due to the shear forces associated with nebulization. There was further reduction in the diameter of liposome particles recovered from the aerosol. This finding is consistent with selection of smaller particles for discharge in aerosol. The lack of crystals suggests that crystals may not nebulize as readily as liposomes. With larger dosages of liposomes, size reduction following nebulization occurred, but particles recovered from aerosol were not reduced in size compared to particles that had been cycled in the reservoir of the nebulizer.

EXAMPLE 3

Kinetics of Lactone Ring Opening

The anti-tumor activity of several of the camptothecins are diminished following dissolution in aqueous media. This is due to a hydrolyzable alpha-hydroxy lactone ring (ring E). The change results from acyl cleavage yielding the biologically inactive carboxylate form of the molecule. The lactone ring form of the drug is sheltered in liposomes (Burke, T. G., *Biochemistry* 1993 32:5352–5364), but the carboxyl form of the drug has high affinity for human serum albumin. This leads to rapid conversion of lactone to carboxylate in the presence of human serum albumin, and thus to loss of anti-cancer activity. Deposition within the lungs on alveolar surfaces where there is little albumin and/or interaction with constituents of the liposomes is clearly a factor in preserving the anti-cancer effect of 9-NC.

EXAMPLE 4

Effect of 9-NC on Growth of Human Breast Cancer Explants in Mice

FIG. 1 shows growth in the area of subcutaneous breast cancer xenografts during treatment with 9-NC-DLPC liposome aerosol. There were six 9-NC treated and 5 control mice. Treatments were given 15 minutes daily, five days per week. The dose was 8.1 μg/kg per day. The deposited dose in the respiratory tract of each mouse was estimated to be 234 ng per day. The data on tumor size was normalized and the divergence of tumor size (% initial tumor growth) in the two groups was highly significant by day 17 of treatment ($P<0.011$). After this time, control mice were sacrificed because of the presence of large necrotic tumor masses. FIG. 2 shows the course of events with two treated mice which were subsequently followed with higher doses of drug, following a period of 16 days without treatment. A few days following restart of treatment with a five-fold increase in the dose of 9-NC liposome aerosol, the size of tumors in the treated animals diminished rapidly, and were no longer visible by the 85th day after start of treatment.

EXAMPLE 5

Effect of 9-NC on Human ColoRectal Cancer Xenografts in Nude Mice

A similar study was performed in nude mice with human colon carcinoma xenografts and is shown in FIG. 3. There were 15 treated and 20 control mice. Ten controls received empty DLPC liposomes and 10 received no treatment. Control animals who received no treatment or DLPC only showed a consistent and rapid increase in tumor size until they were sacrificed on day 36. The overall rate of tumor growth was 7 to 11 times greater in control than in 9-NC-treated mice. The treated animals were divided into two groups of 10 each. One group received 77 μg/kg/daily, five days per week throughout the entire experiment. The other received 77 μg/kg per day five days per week until day 35 when the dose was increased to 153 μg/kg per day five days per week until day 46 when it was increased to 307 μg/kg on the same schedule until day 61. There was slightly less increase in tumor size in the group receiving the higher dose, but the differences were not statistically significant, and the data are combined in the figure. Four mice in the DLPC treatment group were sacrificed because of large tumors or tumor necrosis before day 61, and six mice in the no treatment group were sacrificed for the same reasons before day 61. In the treatment group five mice were sacrificed because of tumor necrosis or emaciation before day 61. The emaciated mice were in the high dose group, suggesting drug toxicity. One additional treated mouse was sacrificed because of rectal prolapse. Based on these findings of reduced rate of tumor growth, day 28, ($P<0.007$, Student t test, 2 tailed) and reduced mortality there is an unequivocal therapeutic effect of 9-NC treatment ($P<0.002$–chi square test).

EXAMPLE 6

Effect of 9-NC on Human Lung Carcinoma Xenografts in Nude Mice

Additionally, studies were performed on the effect of treatment with 9-NC-DLPC via liposome aerosol or via oral administration on the growth of human lung cancer xenografts (Spark) in nude mice as measured by tumor volume. Treatment was initiated about two weeks after tumor implantation. Control animals showed a rapid increase in volume of tumors. Animals who received oral dosage with the liposome drug aqueous suspension in doses of 100 μg/kg/day—more than twice the aerosol dosage—did not respond to treatment. See FIG. 4.

Both aerosol and oral doses were doubled on day 13. The increased dosage was followed by decrease in the size of tumors treated with aerosol, but there was no decrease in size of tumors in mice given oral treatment. Thus, despite the fact that half or more of aerosol dosage administered to mice is deposited in the nose, head, trachea and upper bronchi and is promptly carried by the mucociliary system to the esophagus where it is swallowed, the fraction of inhaled drug that is deposited in the lung is principally responsible for the effect on tumor growth.

The most likely explanation of the clear efficiency of aerosol delivery is the rapid entry of the drug to the circulation where it is returned to the left heart, and then to the aorta and peripheral circulation. Thus, the drug would reach the tumors on "first pass" without having passed through the liver, which would remove large amounts of drug from blood.

EXAMPLE 7

Animal Models

Nude Mice: Swiss immunodeficient nude mice of the NIH-1 high fertility strain, bred and housed at the Stehlin Institute were used for these experiments (Giovella, B. C., et al., Complete Growth Inhibition of Human Cancer Xenografts in Nude Mice by Treatment with 20-(S)-Camptothecin, *Cancer Research* 1991 51:3052–3055).

Human Cancer Xenografts: Human heterotransplants were established in nude mice. For an implant, approximately 50 mg of wet weight of finely minced tumor in 0.5 ml of Eagles minimum essential medium was injected under the skin over the right dorsal chest region. The animals were started on treatment with the experimental drug about 10 days after implantation of tumors. Tumors of breast cancers were measured in two dimensions (i.e. area) with calipers, while colon cancers were measured in three dimensions (i.e. volume) with calipers.

EXAMPLE 8

Camptothecin Liposome Aerosol Formulation and Administration

CPT and 9-nitrocamptothecin were provided by Dr. Beppino Giovanella of the Stehlin Institute, Houston, Tex. DLPC was obtained from Avanti Polar Lipids, Pelham, Ala. Aerotech II nebulizers were obtained from Cis-USA, Inc., Bedford, Mass.

For formulation of liposomes, 9-NC (100 mg/ml) or CPT (10 mg/ml) was dissolved in 100% DMSO, and added to DLPC dissolved in tertiary butanol (40° C.) so the final DMSO concentration did not exceed 5 percent of the total volume and the ratio of drug to lipid was 1:50 (w/w). The final suspension was clear. If precipitation occurred, it was reheated to 50–60° C. The preparation was frozen in liquid nitrogen and lyophilized overnight. For use the material was dissolved in sterile water to the appropriate drug concentration, not exceeding 1.0 mg/ml for either drug. The efficiency of incorporation of drug in the liposomes was examined qualitatively by microscopic examination under polarized light. Unincorporated drug was seen as bi-refringent crystals. The efficiency of incorporation was examined by centrifugation of aqueous suspensions of liposomes on Percoll™ gradients. One-tenth ml of suspension was layered over 2 ml of gradient and centrifuged at 2000 rpm for 25–30 minutes. Liposomes layer at the water-Percoll interface, while unincorporated drug was deposited at the bottom of the tube. Many other lipids may be substituted for DLPC in the formulation and use of liposomes for delivery of drugs by aerosol (Sugarman, S. M., et. al. Lipid-complexed campothecin: formulation and initial biodistribution and anti-tumor activity studies. *Cancer Chemotherapy Pharmacol.* 1996 37:531–538).

EXAMPLE 9

HPLC Analysis

The Waters (Milford, Mass.) 710B Wisp automatic injector and Waters Nova-Pak C18 column at room temperature was used to quantitate CPT and 9-NC. The mobile phase was 30% acetonitrile and 70% of 0.1% glacial acetic acid. CPT was detected using the Waters 470 scanning fluorescence detector set to an excitation wavelength of 370 nm and an emission wavelength of 440 nm. 9-NC was detected using the Waters 440 absorbence detector and monitoring at 254 nm. The data were analyzed with the Waters Millenium software.

EXAMPLE 10

Aerosol Droplet Measurement

The size of aerosol droplets was measured with the Andersen ACFM non-viable ambient particle sizing sampler (Andersen Instruments, Inc., Atlanta, Ga.) by methods previously described (Waldrep, J. C. et. al., *J Aerosol Med.* 1994

7:135–145). Mass median aerodynamic diameters and geometric standard deviations were determined using KaleidaGraph 2.0 (Synergy Software, Reading Pa.). The aerosol droplets consisted of an aqueous suspension of liposomes. Liposome diameters were measured in aqueous suspension with the Model 3300 NICOMP Laser Particle Sizer.

EXAMPLE 11

Preparation and Efficiency of Taxol-DLPC Liposomes Formulated by Aerosol Delivery

Taxol is another anti-cancer drug that is lipid soluble and is incorporated easily into liposomal formulation. Taxol is dissolved directly in t-butanol without use of DMSO similar to the camptothecins. The taxol liposomal preparation is otherwise like that of the camptothecins.

The optimal taxol to DLPC ratio was found to be about 1:30 wt:wt. Formulations compatible with nebulization and aerosol delivery formulations were produced at 1 mg Taxol and with 30 m g DLPC per ml. For optimized Taxol-DLPC liposomes, 5 mg of Taxol was mixed with 150 mg of synthetic alpha-lecithin: dilauroylphosphatidylcholine (DLPC). Working at 37° C., the drug/DLPC was mixed in 20 mls of tertiary butanol with stirring. After mixing, the drug/lipid preparation was pipetted into glass vials, frozen rapidly, and lyophilized overnight to remove the t-butanol leaving a powder. Multi-lamellar liposomes were produced by adding 5 mls of ultra pure water above the DLPC phase transition temperature (Tc) at 25° C. to deliver a final standard drug concentration of 1 mg Taxol: 30 mg DLPC per ml. The mixture was incubated for 30 minutes at room temperature with intermittent mixing to produce multilamellar vesicular (MLV) liposomes. Aliquots were removed for determination of drug concentration by HPLC.

FIG. 5 shows the particle size distribution of taxol-DLPC liposome aerosol with a MMAD of 1.4 μm and a geometric standard deviation of 2.0 FIG. 6 shows the recovery of taxol from the preparation described in FIG. 5. In the lung model (Harvard Respirator) used to measure the output of taxol from the nebulizer (Aerotech II), 87.75 liters of aerosol were sampled yielding 3000 μg of taxol. There was thus an aerosol concentration of 34.2 μg/l. From this information, the dose of aerosol deposited in the respiratory tract following inhalation can be calculated.

Any patents or publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. Further, these patents and publications are incorporated by reference herein to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

One skilled in the art will appreciate readily that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those objects, ends and advantages inherent herein. The present examples, along with the methods, procedures, treatments, molecules, and specific compounds described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention as defined by the scope of the claims.

What is claimed is:

1. A method for treating cancer, comprising the step of delivering, via small particle aerosol, aqueous dispersions of anti-cancer drugs in liposomes to the respiratory tract of an individual in need of such treatment, wherein said anti-cancer drug is selected from the group consisting of taxol, taxol-A, mitotane, methotrexate, mercaptopurine, lomustine, interferon, 5-fluorouracil and etopside and wherein a final concentration of said anticancer drug in said liposomes is no greater than 5.0 mg/ml.

2. The method of claim 1, wherein said delivering step is performed by a jet nebulizer.

3. The method of claim 2, wherein said liposomes are sheared to a diameter of less than 500 nm by said jet nebulizer.

4. The method of claim 1, wherein a final concentration of said anticancer drug in said liposomes is no greater than 1.0 mg/ml.

* * * * *

Applicants

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[45] **Date of Patent: May 5, 1998**

[54] **METHODS AND COMPOSITIONS COMPRISING DNA DAMAGING AGENTS AND P53**

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[58] **Field of Search** 514/44; 435/91, 435/93.21, 172.1, 172.3, 240.1, 240.2, 320.1, 91.31; 424/93.2, 93.21

[56] **References Cited**

U.S. PATENT DOCUMENTS

4,740,463	4/1988	Weinberg et al.	435/172.3
4,980,289	12/1990	Temin et al.	435/235.1
5,055,400	10/1991	Lo et al.	435/69.1
5,166,320	11/1992	Wu et al.	530/395
5,252,479	10/1993	Srivastava	435/235.1
5,328,470	7/1994	Nabel et al.	60/101
5,532,220	7/1996	Lee et al.	514/44

FOREIGN PATENT DOCUMENTS

0174608	9/1985	European Pat. Off. .
0351585	6/1989	European Pat. Off. .
0 390 323	10/1990	European Pat. Off. .
0475623	8/1991	European Pat. Off. .
2688514	9/1993	France .
WO 90/10448	9/1990	WIPO .
WO 93/03769	3/1993	WIPO .
9408026	4/1994	WIPO .
WO 94/10323	5/1994	WIPO .
WO 94/24297	10/1994	WIPO .
WO 95/02697	1/1995	WIPO .

OTHER PUBLICATIONS

Baker, Suzanne, J., et al., "Suppression of Human Colorectal Carcinoma Cell Growth by Wild-Type p53", *Science*, vol. 249, pp. 912–915, Aug. 24, 1990.

Bargonetti, Jill, et al., "Wild-Type but Not Mutant p53 Immunopurified Proteins Bind to Sequences Adjacent to the SV40 Origin of Replication", *Cell*, vol. 65, pp. 1083–1091, Jun. 14, 1991.

Berkner, Kathleen L., "Development of Adenovirus Vectors for the Expression of Heterologous Genes", *BioTechniques*, vol. 6, No. 7, pp. 616–629, 1988.

Blenis, John, "Signal transduction via the MAP kinases: Proceed at your own RSK", *Proc. Natl. Acad. Sci. USA*, vol. 90, pp. 5889–5892, Jul. 1993.

Brachman, David G., et al., "p53 Mutation Does Not Correlate with Radiosensitivity in 24 Head and Neck Cancer Cell Lines", *Cancer Research*, vol. 53, pp. 3667–3669, Aug. 15, 1993.

Capecchi, Mario R., "Altering the Genome by Homologous Recombination", *Science*, vol. 244, pp. 1288–1292, Jun. 16, 1989.

Cheng, Jian, et al., "Suppression of Acute Lymphoblastic Leukemia by the Human Wild-Type p53 Gene", *Cancer Research*, vol. 52, pp. 222–226, Jan. 1, 1992.

Coleman, C. Norman and Turrisi, Andrew T., "Radiation and Chemotherapy Sensitizers and Protectors", *Critical Reviews In Oncology/Hematology*, vol. 10, Issue 3, pp. 225–252, 1990.

Comings, David E., "A General Theory of Carcinogenesis", *Proc. Natl. Acad. Sci. USA*, vol. 70, No. 12, Part I, pp. 3324–3328, Dec. 1973.

Friedman, M.D., Theodore, "Gene Therapy of Cancer through Restoration of Tumor-Suppressor Functions?", *Cancer* Supplement, vol. 70, No. 6, pp. 1810–1817, Sep. 15, 1992.

Gudkov, Andrei V., et al., "Isolation of genetic suppressor elements, inducing resistance to topoisomerase II–interaction cytotoxic drugs, from human topoiosmerase II cDNA", *Proc. Natl. Acad. Sci. USA*, vol. 90, pp. 3231–3235, Apr. 1993.

Houghten, Richard A., "Peptide libraries: criteria and trends", *Technical Focus*, vol. 9, No. 7 pp. 235–239, Jul. 1993.

Itoh, Naoto, et al., "The Polypeptide Encoded by the cDNA for Human Cell Surface Antigen Fas Can Mediate Apoptosis", *Cell*, vol. 66, pp. 233–243, Jul. 26, 1991.

(List continued on next page.)

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[57] **ABSTRACT**

The present invention relates to the use of tumor suppressor genes in combination with a DNA damaging agent or factor for use in killing cells, and in particular cancerous cells. A tumor suppressor gene, p53, was delivered via a recombinant adenovirus-mediated gene transfer both in vitro and in vivo, in combination with a chemotherapeutic agent. Treated cells underwent apoptosis with specific DNA fragmentation. Direct injection of the p53-adenovirus construct into tumors subcutaneously, followed by intraperitoneal administration of a DNA damaging agent, cisplatin, induced massive apoptotic destruction of the tumors. The invention also provides for the clinical application of a regimen combining gene replacement using replication-deficient wild-type p53 adenovirus and DNA-damaging drugs for treatment of human cancer.

105 Claims, 22 Drawing Sheets

OTHER PUBLICATIONS

Jayawickreme, Channa K., et al., "Creation and functional screening of a multi-use peptide library", *Proc. Natl. Acad. Sci. USA*, vol. 91, pp. 1614–1618, Mar. 1994.

Kamb, Alexander, et al., "A Cell Cycle Regulator Potentially Involved in Genesis of Many Tumor Types", *Science*, vol. 264, pp. 436–440, Apr. 15, 1994.

Kern, Scott E., et al., "Identification of p53 as a Sequence-Specific DNA-Binding Protein", *Science*, vol. 252, pp. 1708–1711, Jun. 21, 1991.

Knudson, Jr., Alfred G., "Mutation and Cancer: Statistical Study of Retinoblastoma", *Proc. Natl. Acad. Sci. USA*, vol. 68, No. 4, pp. 820–823, Apr. 1971.

Kuerbitz, Steven, J., et al., "Wild-type p53 is a cell cycle checkpoint determinant following irradiation", *Proc. Natl. Acad. Sci. USA*, vol. 89, pp. 7491–7495, Aug. 1992.

Li, Frederick P., et al., "A Cancer Family Syndrome in Twenty-four Kindreds", *Cancer Research*, vol. 48, pp. 5358–5362, Sep. 15, 1988.

Malkin, David, et al., "Germ Line p 53 Mutations in a Familial Syndrome of Breast Cancer, Sarcomas, and Other Neoplasms", *Science*, vol. 250, pp. 1233–1238, Nov. 30, 1990.

Mansour, Suzanne L., et al., "Introduction of a lacZ reporter gene into the mouse int-2 locus by homologous recombination", *Proc. Natl. Acad. Sci. USA*, vol. 87, pp. 7688–7692, Oct. 1990.

Marshall, C. J., "Hot lips and phosphorylation of protein kinases", *Nature*, vol. 367, p. 686, Feb. 24, 1994.

Mercer, W. Edward, et al., "Negative growth regulation in a glioblastoma tumor cell line that conditionally expresses human wild-type p53", *Proc. Natl. Acad. Sci. USA*, vol. 87, pp. 6166–6170, Aug. 1990.

Michalovitz, Dan, et al. "Conditional Inhibition of Transformation and of Cell Proliferation by a Temperature-Sensitive Mutant of p53", *Cell*, vol. 62, pp. 671–680, Aug. 24, 1990.

Nigro, Janice M., et al., "Mutations in the p53 gene occur in diverse human tumour types", *Nature*, vol. 342, pp. 705–708, Dec. 7, 1989.

O'Connor, Patrick M., et al., "Role of the p53 Tumor Suppressor Gene in Cell Cycle Arrest and Radiosensitivity of Burkitt's Lymphoma Cell Lines", *Cancer Research*, vol. 53, pp. 4776–4780, Oct. 15, 1993.

Paull, Kenneth D., et al., "The Synthesis of XTT: A New Tetrazolium Reagent that is Bioreducible to a Water-Soluble Formazan", *J. Heterocyclic Chem.*, vol. 25, pp. 911–914, May–Jun. 1988.

Petty, Russell D., et al., "Expression of the p53 Tumour Suppressor Gene Product is a Determinant of Chemosensivity", *Biochem. and Biophys. Research Communications*, vol. 199, No. 1, pp. 264–270, Feb. 28, 1994.

Phillips, Margaret, A., et al., "Transition-State Characterization: A New Approach Combining Inhibitor Analogues and Variation in Enzyme Structure", *Biochemistry*, vol. 31, pp. 959–963, 1992.

Revet, Bernard M. J., et al., "Homologous DNA Targeting with RecA Protein-coated Short DNA Probes and Electron Microscope Mapping on Linear Duplex Molecules", *J. Mol. Biol.*, vol. 232, pp. 779–791, 1993.

Saris, M.D., Stephen C., et al., "Treatment of murine primary brain tumors with systemic interleukin-2 and tumor-infiltrating lyphocytes", *J. Neurosurg.*, vol. 76, pp. 513–519, 1992.

Shaulsky, Gad, et al., "Involvement of wild-type p53 in pre-B-cell differentiation in vitro", *Proc. Natl. Acad. Sci. USA*, vol. 88, pp. 8982–8986, Oct., 1991.

Shay, Jerry W., et al., "A Role for Both RB and p53 in the Regulation of Human Cellular Senescence", *Experimental Cell Research*, vol. 196, pp. 33–39, 1991.

Muzyczka, N., "Use of Adeno-Associated Virus as a General Transduction Vector for Mammalian Cells", *Current Topics in Microbiol. and Immunol.*, vol. 158, pp. 98–129, 1992.

Srivastava, Shiv., et al., "Germ-line transmission of a mutated p53 gene in a cancer-prone family with Li-Fraumeni syndrome", *Nature*, vol. 348, pp. 747–749, Dec. 1990.

Takahashi, Takashi, et al., "p53: A Frequent Target for Genetic Abnormalities in Lung Cancer", *Science*, vol. 246, pp. 491–494, Oct. 27, 1989.

Thomas, Kirk R., et al., "High-Fidelity Gene Targeting in Embryonic Stem Cells by Using Sequence Replacement Vectors", *Molecular and Cellular Biol.*, vol. 12, No. 7, pp. 2919–2923, Jul. 1992.

Ullrich, Stephen J., et al., "Human wild-type p53 adopts a unique conformational and phosphorylation state in vivo during growth arrest of glioblastoma cells", *Oncogene*, vol. 7, pp. 1635–1643, 1992.

Unger, Tamar, et al., "Functional Domains of Wild-Type and Mutant p53 Proteins Involved in Transcriptional Regulation, Transdominant Inhibition, and Transformation Suppression", *Molecular and Cellular Biol.*, vol. 13, No. 9, pp. 5186–5194, Sep., 1993.

Vogelstein, Bert and Kinzler, Kenneth W., "p53 Function and Dysfunction", *Cell*, vol. 70, pp. 523–526, Aug. 21, 1992.

Wagner, Ernst, et al., "Coupling of adenovirus to transferrin-polylysine/DNA complexes greatly enhances receptor-mediated gene delivery and expression of transfected genes", *Proc. Natl. Acad. Sci. USA*, vol. 89, pp. 6099–6103, Jul. 1992.

Weislow, Owen S., et al., "New Soluble-Formazan Assay for HIV-1 Cytopathic Effects: Application to High-Flux Screening of Synthetic and Natural Products for AIDS-Antiviral Activity", *J. Natl. Cancer Inst.*, vol. 81, No. 8, pp. 577–586, Apr. 19, 1989.

Wu, George Y., et al., "Receptor-mediated Gene Delivery in vivo", *The J. of Biol. Chem.*, vol. 266, No. 22, pp. 14338–14342, Aug. 5, 1991.

Yonish-Rouach, Elisheva, et al., "Wild-type p53 induces apoptosis of myeloid leukemic cells that is inhibited by interleukin-6", *Nature*, vol. 352, pp. 345–347, Jul. 25, 1991.

Yoshimura, Kunihiko, et al., "Expression of the human cystic fibrosis transmembrane conductance regulator gene in the mouse lung after in vivo intratracheal plasmid-mediated gene transfer", *Nucleic Acid Research*, vol. 20, No. 12, pp. 3233–3240, Apr. 1992.

Zhu, Ning, et al., "Systemic Gene Expression After Intravenous DNA Delivery into Adult Mice", *Science*, vol. 261, pp. 209–211, Jul. 9, 1993.

Singleton, Paul and Sainsbury, Diana, *Dictionary of Microbiology and Molecular Biology*, 2nd Ed., John Wiley & Sons, Publ., pp. 591 and 920.

Stedman's Medical Dictionary, 25th Ed., Williams & Wilkins, Publ., p. 245.

Comprehensive Textbook of Oncology, vol. 1, 2nd Ed., Williams & Wilkins, Publ., pp. 477, 527–536, 565–568, 590–594, 607–612.

Comprehensive Textbook of Oncology, vol. 2, 2nd Ed., Williams & Wilkins, Publ., pp. 1098, 1138–1140, 1170, 1329, 1368, 1569–1572.

Federal Register, vol. 47, No. 56, pp. Title –VI and i–iv, Mar. 23, 1982.

Steel, Michael, "Cyclins and cancer: wheels within wheels", *The Lancet*, vol. 343, pp. 931–932, Apr. 16, 1994.

Proceedings of the American Association for Cancer Research, vol. 36, p. 21, Mar. 1995.

Fujiwara, Toshiyoshi, et al., "Induction of Chemosensitivity in Human Lung Cancer Cells in vivo by Adenovirus–mediated Transfer of the Wild–Type p53 Gene", *Cancer Research*, vol. 54, pp. 2287–2291, May 1, 1994.

Diller, Lisa, et al., "p53 Functions as a Cell Cycle Control Protein in Osteosarcomas", Molecular and Cellular Biology, vol. 10, No. 11, pp. 5772–5781, Nov. 1990.

Cureil, et al., 3, "High–efficiency gene transfer mediated by adenovirus coupled to DNA–polylysine complexes", *Human Gene Therapy*, 147–154 (1992).

Foreman, et al., 4(3) Title Unknown, *Bone Marrow Transportation* (1990).

Graham, et al., 7 Title Unknown, *Methods in Molecular Biology, Gene Transfer and Expression Protocols*, Ch. 11, pp. 109–128 (1991).

Hinds, et al., *Cell Growth and Differentiation*, 571–580 (1990).

Huston, et al., 10(2–3) "Medical applications of single–chain antibodies", *International Reviews of Immunology*, 195–217 (1993).

Jolly, D., 1(1) "Viral vector systems for gene therapy", *Cancer Gene Therapy*, 51–64 (1994).

Kriegler, et al., *Gene Transfer and Expression: A Laboratory Manual*.

Romer, K. and Friedman, T., 716 "Annals of the New York Academy of Science, Gene Therapy for Neoplastic Diseases" 265–282 (1994).

Van de Waterbeemd, 9 "Recent progress in QSAR–technology", *Drug Design and Discovery* 277–285 (1993).

Su et al., "Transformation and radiosensitivity of human diploid skin fibroblasts transfected with SV40 T–antigen mutants defective in RB and P53 binding domains," *Int. J. Radiat. Biol.*, 1992 vol. 62, No. 4, 461–468.

Petty et al., "Expression of the p53 Tumour Suppressor Gene Product is a Determinant of Chemosensitivity," *Biochemical and Biophysical Research Communications*, 199(1):264–270, Feb. 1994.

PCT Search Report dated Apr. 24, 1995.

Brown et al., "Mutant p53 Confers Cisplatin–sensitivity to Resistant Ovarian Tumour Cells with Elevated Wild–Type p53," *Proc. Am. Assoc. Cancer Res.*, 34:355, #2116, 1993.

Donehower, "Tumor Suppressor Gene p53 and Apoptosis," *Cancer Bulletin* 46(2):161–166, 1994.

El Rouby et al., "p53 gene mutation in B–cell chronic lymphocytic leukemia is associated with drug resistance and is independent of MDR1/MDR3 gene expression," *Blood*, 82(11):3452–3459, 1993.

Fan et al., "The role of p53 in cell cycle arrest and apoptosis induced by multiple chemotherapeutic agents in Burkitt's lymphoma cells," *Proceedings of the American Association for Cancer Research*, 35:311, #1851, 1994.

Fornace, Jr. "Induction by radiation of mammalian genes associated with growth–arrest and apoptosis, and the role of the p53 tumor suppressor in their regulation," *Proceedings of the American Association for Cancer Research*, 35:682–682, 1994.

Fritsche et al., "Induction of nuclear accumulation of the tumor–suppressor protein p53 by DNA–damaging agents", published erratum, *Oncogene*, 8(9):2605, 1993.

Hecht et al., "Comparison of wildtype and mutated p53 protein expression induced by UV irradiation of cultured cells," *FASEB Journal*, 8:A667, #3870, 1994.

Kaneko et al., "Induction of apoptosis and p53 protein by adriamycin and hyperthermia in a rat mammary adenocarcinoma cell line," *Proceedings of the American Association for Cancer Research*, 35:314, #1871, 1994.

Kastan et al., "Participation of p53 protein in the cellular response to DNA damage," *Cancer Res.*, 51:6304–11, 1991.

Kastan, "p53: a determinant of the cell cycle response to DNA damage," *Adv. Exp. Med. Biol.*, 339:295–296, 1993.

Kastan et al., "p53 and other molecular controls of the response to DNA damage," *Journal of Cellular Biochemistry*, 9(18C):164, 1994.

Kemp et al., "p53–deficient mice are extremely susceptible to radiation–induced tumorigenesis," *Nature Genetics*, 8(1):66–69, 1994.

Lane, "A death in the life of p53," *Nature*, 362:786–787, 1993.

Lee & Bernstein, "p53 mutations increase resistance to ionizing radiation," *Proc. Natl. Acad. Sci. USA*, 90(12):5742–5746, 1993.

Levine et al., "The 1993 Walter Hubert Lecture: the role of the p53 tumour–suppressor gene in tumorigenesis," *Br. J. Cancer*, 69(3):409–416, 1994.

Loganzo, Jr. et al., "Stabilization of p53 protein is a critical response to UV radiation in human melanocytes: Implications for melanoma development," *Molecular and Cellular Differentiation*, 2(1):23–43, 1994.

Lotem & Sachs, "Regulation by bcl–2, c–myc and p53 of susceptibility to induction of apoptosis by heat shock and cancer chemotherapy compounds in differentiation–competent and –defective myeloid leukemic cells," *Cell Growth Differ.*, 4(1):41–7, 1993.

Lotem & Sachs, "Hematopoietic cells from mice deficient in wild–type p53 are more resistant to induction of apoptosis by some agents," *Blood*, 82(4):1092–6, 1993.

Maity et al., "The molecular basis for cell cycle delays following ionizing radiation: a review," *Radiother. Oncol.*, 31(1):1–13, 1994.

McIlwrath et al., "Cell cycle arrests and radiosensitivity of human tumor cell lines: Dependence on wild–type p53 for radiosensitivity," *Cancer Research*, 54(14):3718–3722, 1994.

Nabeya et al., "The mutational status of p53 protein in gastric cancer cell lines predicts sensitivity to chemotherapeutic agents," *Proceedings of the American Association for Cancer Research*, 35:602, #3591, 1994.

O'Connor et al., "Relationship between p53, cyclin E–cdk2 kinase complexes and G1 arrest induced by ionizing radiation in human cells," *Proceedings of the American Association for Cancer Research*, 35:635, #3785, 1994.

Rau et al., "Response of p53 to treatment with actinomycin D in human mammary carcinoma cell lines," *Journal of Cancer Research and Clinical Oncology*, 120:R108, 1994.

Shaw et al., "Induction f apoptosis by wild–type p53 in a human colon tumor–derived cell line," *Proc. Natl. Acad. Sci. USA*, 89(10):4495–4499, 1992.

Slichenmyer et al., "Loss of a p53–associated G1 checkpoint does not decrease cell survival following DNA damage," *Cancer Res.*, 53(18):4164–4168, 1993.

Varghese et al., "The role of p53 and ras genes in radiation–induced transformation of immortalized human epidermal keratinocytes." *Proceedings of the American Association for Cancer Research*, 35:91, #542, 1994.

Yamada & Ohyama, "Radiation and apoptosis," *Gan To Kagaku Ryoho*, 21(5):602–607, 1994, Abstract only.

Bandyopadhyay & Temin, "Expression of Complete Chicken Thymidine Kinase Gene Inserted in a Tetrovirus Vector." *Molecular and Cellular Biology*, 4(4):749–754, 1984.

Bowtell et al., "Comparison of Expression in Hemopoietic Cells by Retroviral Vectors Carrying Two Genes," *Journal of Virology*, 62(7):2464–2473, 1988.

Casson et al., "p53 Gene Mutations in Barrett's Epithelium and Esophageal Cancer", *Cancer Research*, 51:4495–4499, 1991.

Chen et al., "Genetic Mechanisms of Tumor Suppression by the Human p53 Gene," *Science*, 250:1576–1580, 1990.

Chen et al., "Expression of Wild–Type p53 in Human A673 Cells Suppresses Tumorigenicity but Not Growth Rate," *Oncogene*, 6:1799–1805, 1991.

Goyette et al., "Progression of Colorectal Cancer Is Associated with Multiple Tumor Suppressor Gene Defects but Inhibition of Tumorigenicity Is Accomplished by Correction of Any Single Defect via Chromosome Transfer," *Molecular and Cellular Biology*, 12(3):1387–1395, 1992.

Gusterson et al., "Expression of p53 in Premalignant and Malignant Squamous Epithelium," *Oncogene*, 6:1785–1798, 1991.

Kumar et al., "Activation of ras Oncogenes Preceding the Onset of Neoplasia," *Science*, 248:1101–1104, 1990.

Maxwell et al., "Inefficiency of Expression of Luciferase Reporter from Transfected Murine Leukaemia Proviral DNA May Be Partially Overcome by Providing a Strong Polyadenylation Signal," *Journal of General Virology*, 72:1721–1724, 1991.

Mukhopadhyay et al., "Specific Inhibition of K–ras Expression and Tumorigenicity of Lung Cancer Cells by Antisense RNA," *Cancer Research*, 51:1744–1748, 1991.

Owens & Boyd, "Expressing Antisense Po RNA in Schwann Cells Perturbs Myelination," *Development*, 112:639–649, 1991.

Palmer et al., "Efficient Retrovirus–Mediated Transfer and Expression of a Human Adenosine Deaminase Gene in Diploid Skin Fibroblasts from an Adenosine Deaminase–Deficient Human," *Proceedings of the National Academy of Science USA*, 84:1055–1059, 1987.

Seyama et al., "In Vitro and In Vivo Regulation of Liver Epithelial Cells Carrying a Metallothionein–rasT24 Fusion Gene," *Molecular Carcinogenesis*, 1:89–95, 1988.

Takahashi et al., "Wild–Type but Not Mutant p53 Suppresses the Growth of Human Lung Cancer Cells Bearing Multiple Genetic Lesions," *Cancer Research*, 52:2340–2343, 1992.

Zhou & Duesberg, "myc Protooncogene Linked to Retroviral Promoter, but Not to Enhancer, Transforms Embryo Cells," *Proceedings of the National Academy of Science USA*, 85:2924–2928, 1988.

Conroy, "New Gene Therapy Cleared for Use Against Lung Cancer," *Biotech Daily*, pp. 3–4, Sep. 18, 1992.

Dialog Search Report dated Sep. 22, 1992.

Sundaresan, V., et al., "Somatic Genetic Changes in Pre–Invasive Lesions in Bronchial Epithelium," *J. Pathol.*, 167(Suppl) 1992, 100A, Abstract Only.

Dialog Search Reports dated Aug. 7, 1992 and Feb. 26, 1993.

Debus et al., J Cancer Res Clin Oncol, 116 (Suppl Part 1):5–162, Abstract # A2.037.09, 1990.

Delauney et al., "A stable bifunctional antisense transcript inhibiting gene expression in transgenic plants", *Proc. Natl. Acad. Sci. USA*, 85:4300–4304, 1988.

Feig, et al., "Somatic Activation of ras^ Gene in a Human Ovarian Carcinoma", *Science*, 223:698–701, 1984.

Finkel, et al., "Activation of ras Genes in Human Tumors Does Not Affect Localization, Modification, or Nucleotide Binding Properties of p21", *Cell*, 37:151–158, 1984.

Griep and Heiner, "Antisense Myc sequences induce differentiation of F9 cells", *Proc. Natl. Acad. Sci. USA*, 85:6806–6810, 1988.

Gunning, et al., "A human β–actin expression vector system directs high–level accumulation of antisense transcripts", *Proc. Natl. Acad. Sci. USA*, 84:4831–4835, 1987.

Kasid, et al., "Effect of Antisense c–raf–1 on Tumorigenicity and Radiation Sensitivity of a Human Squamous Carcinoma", *Science*, 243:1354–1356, 1989.

Khokha, Rama, et al., "Antisense RNA–Induced Reduction in Murine TIMP Levels Confers Oncogenicity on Swiss 3T3 Cells", *Science*, 243:947–950, 1989.

Kris, et al. "Expression of Ki–Ras Oncogene in Tumor Cell Variants Exhibiting Different Metstatic Capabilities", *Int. J. Cancer*, 35:227–230, 1985.

Izant and Weintraub, "Inhibition of Thymidine Kinase Gene Expression by Anti–Sense RNA: A Molecular Approach to Genetic Analysis", *Cell*, 36:1007–1015, 1984.

Johnson, et al., "Transfection of a Rat Cell Line with the v–Ki–ras Oncogene is Associated with Enhanced Susceptibility to Natural Killer Cell Lysis", *J. Exp. Med.*, 162:1732–1737, 1985.

McGrath, et al., "Structure and organization of the human Ki–ras protooncogene and a related processed pseudogene", *Nature*, 304:501, 1983.

Magrath, "Tumor–specific antisense oligonucleotides for controlling cancer", Abstract No. 114:55778n, *Chemical Abstracts*, 114(7):68 (1991).

Mercola, et al., "Antisense RNA: Eukaryotic Controls", *Gene*, 72:253–265 (1988).

Miller and Rosman, Improved Retroviral Vectors for Gene Transfer and Expression, *BioTechniques*, 7(9):980–990, 1989.

Munroe, Stephen H., "Antisense RNA inhibits splicing of pre–mRNA in vitro", *The EMBO Journal*, 7(8):2523–2532 (1988).

Prochownik, et al., "c–myc Antisense Transcripts Accelerate Differentiation and Inhibit G_1 Progression in Murine Erythroleukemia Cells", *Molecular and Cellular Biology*, 8(9):3683–3695, 1988.

Santos, et al., Malignant Activation of a K–ras Oncogene in Lung Carcinoma but Not in Normal Tissue of the Same Patient, *Science*, 223:661–664, 1984.

Shimizu, et al., "Structure of the Ki–ras gene of the human lung carcinoma cell line Calu–1", *Nature*, 304:497–500, 1983.

Stowers, et al., "Activation of the K–ras Protooncogene in Lung Tumors from Rats and Mice Chronically Exposed to Tetranitromethane", *Cancer Research*, 47:3212–3219, 1987.

Taya, et al., "A novel combination of K–ras and myc amplification accompanied by point mutational activation of K–ras in a human lung cancer", *The EMBO Journal*, 3(12):2943–2946, 1984.

Toftgard, et al., "Proto-oncogene expression during two-stage carcinogenesis in mouse skin", *Carcinogenesis*, 6(4):655–657, 1985.

Vogelstein, et al., "Genetic Alterations During Colorectal-Tumor Development", *The New England Journal of Medicine*, 319(9):525–532, 1988.

Wahran et al., Tumour Biol. 6:41–56, 1985.

Winter and Perucho, "Oncogene Amplification during Tumorigenesis of Established Rat Fibroblasts Reversibly Transformed by Activated Human ras Oncogenes", *Molecular and Cellular Biology*, 6(7):2562–2570, 1986.

International Search Report, mailed Aug. 20, 1992.

Brown et al., "Increased Accumulation of p53 Protein in Cisplatin-Resistant Ovarian Cell Lines," *Int. J. Cancer*, 55:678–684, 1993.

Clarke et al., "Thymocyte apoptosis induced by p53-dependent and independent pathways," *Nature*, 362:849–852, 1993.

El-Deiry et al., "WAF1, a Potential Mediator of p53 Tumor Suppression," *Cell*, 75:817–825, 1993.

Fritsche et al., "Induction of nuclear accumulation of the tumor-suppressor protein p53 by DNA-damaging agents," *Oncogene*, 8:307–318, 1993.

Fujiwara et al., "A Retroviral Wild-type p53 Expression Vector Penetrates Human Lung Cancer Spheroids and Inhibits Growth by Inducing Apoptosis," *Cancer Research*, 53:4129–4133, 1993.

Harper et al., "The p21 Cdk-Interacting Protein Cip1 Is a Potent Inhibitor of G1 Cyclin-Dependent Kinases," *Cell*, 75:805–816, 1993.

Lowe et al., "p53-Dependent Apoptosis Modulates the Cytotoxicity of Anticancer Agents," *Cell*, 74:957–967, 1993.

Lowe et al., "p53 is required for radiation-induced apoptosis in mouse thymocytes," *Nature*, 362:847–849, 1993.

Merritt et al., "The role of p53 in Spontaneous and Radiation-induced Apoptosis in the Gastrointestinal Tract of Normal and p53-deficient Mice," *Cancer Research*, 54:614–617, 1994.

Tishler et al., "Increases in Sequence Specific DNA Binding by p53 following Treatment with Chemotherapeutic and DNA Damaging Agents," *Cancer Research*, 53:2212–2216, 1993.

Casey et al., "Growth Suppression of Human Breast Cancer Cells by the Introduction of a Wild-Type p53 Gene," *Oncogene*, 6:1791–1797, 1991.

Wills and Menzel, "Adenovirus Vectors for Gene Therapy of Cancer," *Journal of Cellular Biochemistry*, p. 204, Abstract # S216, Mar.–Apr. 1993.

Zhang et al., "Generation and Identification of Recombinant Adenovirus by Liposome-Mediated Transfection and PCR Analysis," *BioTechniques*, 15(5):868–872, 1993.

PCT Search Report dated Jul. 5, 1995.

Marshall, Science, vol. 269, 1995 pp. 1050–1055.

Neve, Trends Neurosci, vol. 16(7), 1993, pp. 251–253.

Bachetti et al, Int. J. Oncol. vol. 3(5), 1993, pp. 781–788.

Tishler et al, Cancer Res., vol. 53, 1993 pp. 2212–2216.

Culver et al, Science, vol. 256, 1992 pp. 1550–1552.

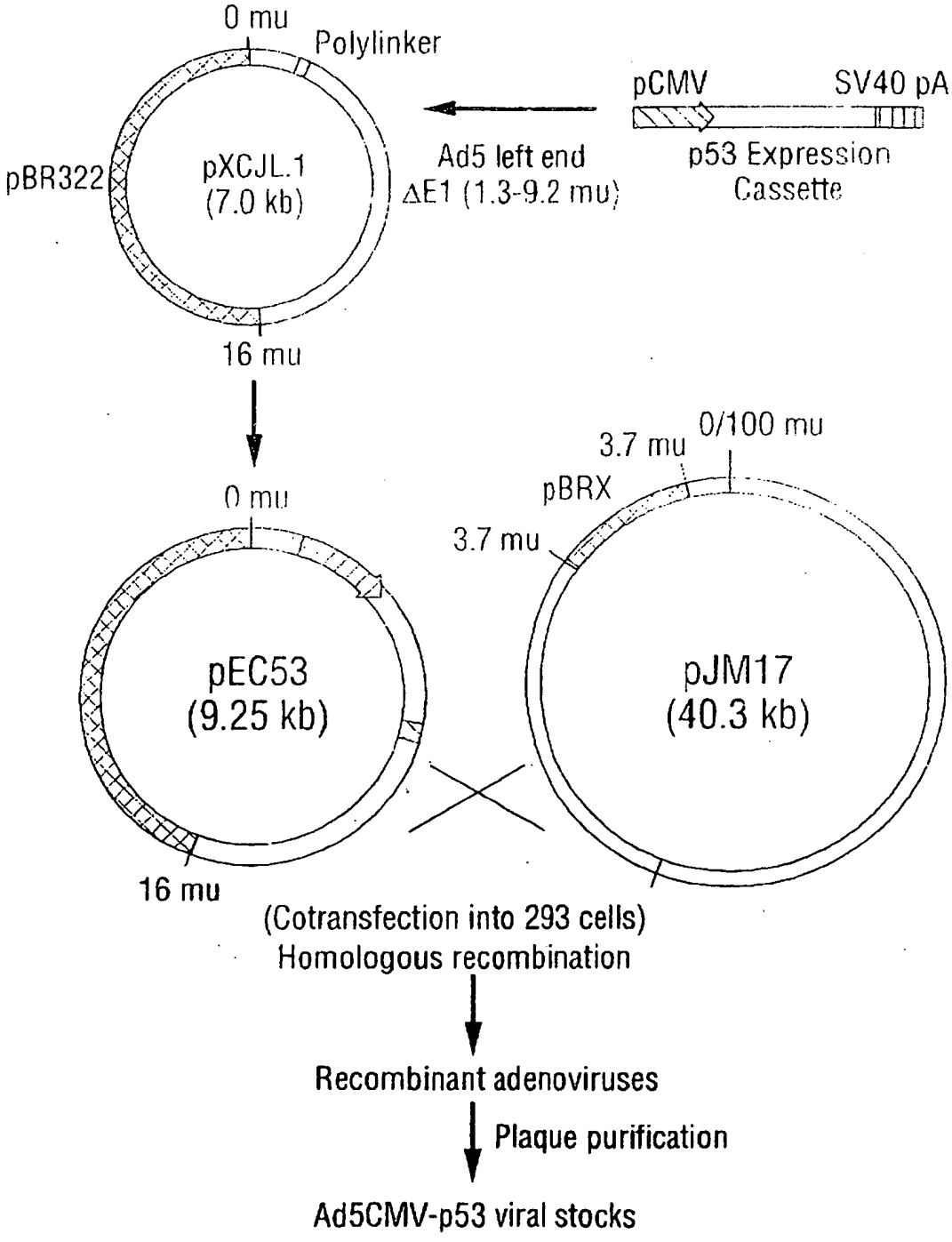
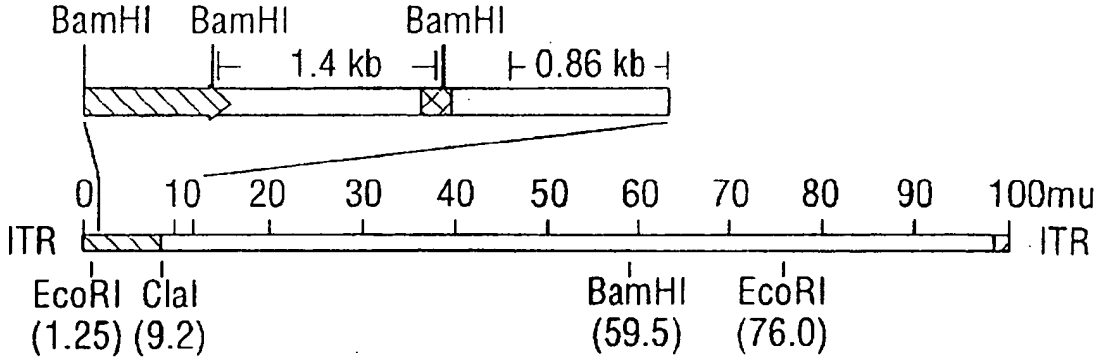

0 mu
Polylinker
pBR322
pXCJL.1
(7.0 kb)
16 mu
Ad5 left end
ΔE1 (1.3-9.2 mu)
pCMV
SV40 pA
p53 Expression
Cassette
0 mu
pEC53
(9.25 kb)
16 mu
3.7 mu
0/100 mu
pBRX
3.7 mu
pJM17
(40.3 kb)
(Cotransfection into 293 cells)
Homologous recombination
Recombinant adenoviruses
Plaque purification
Ad5CMV-p53 viral stocks

FIG. 1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BamHI
BamHI
1.4 kb
0.86 kb
0
10
20
30
40
50
60
70
80
90
100mu
ITR
ITR
EcoRI
(1.25)
ClaI
(9.2)
BamHI
(59.5)
EcoRI
(76.0)

FIG. 2A

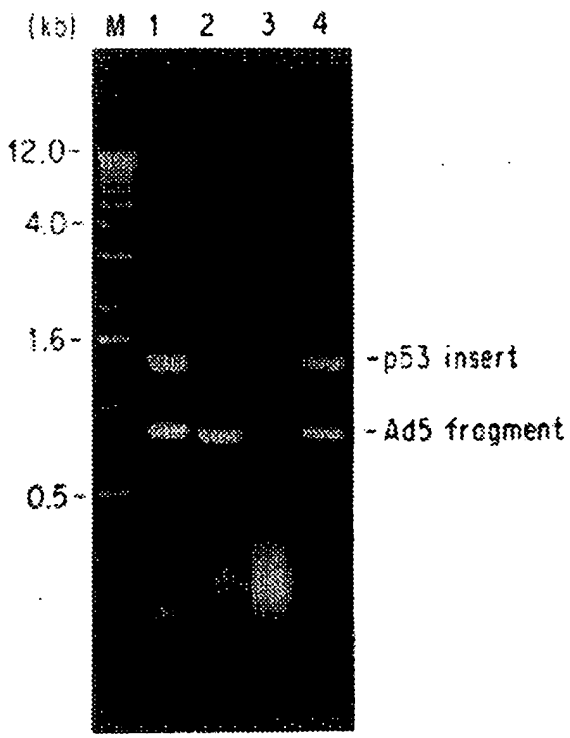

(kb) M 1 2 3 4
12.0
4.0
1.6
0.5
p53 insert
Ad5 fragment

FIG.2B

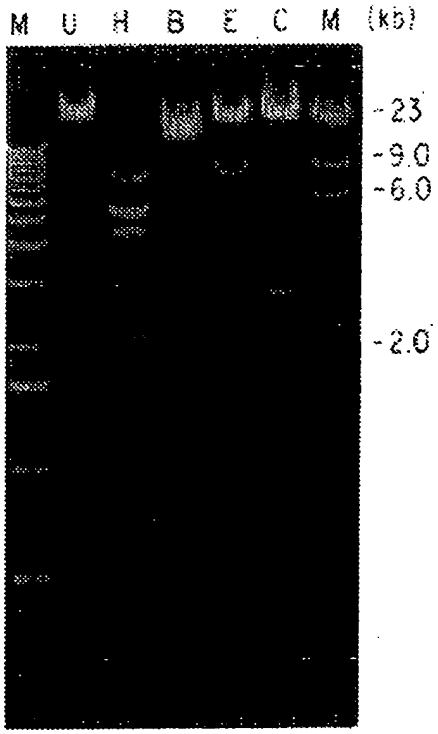
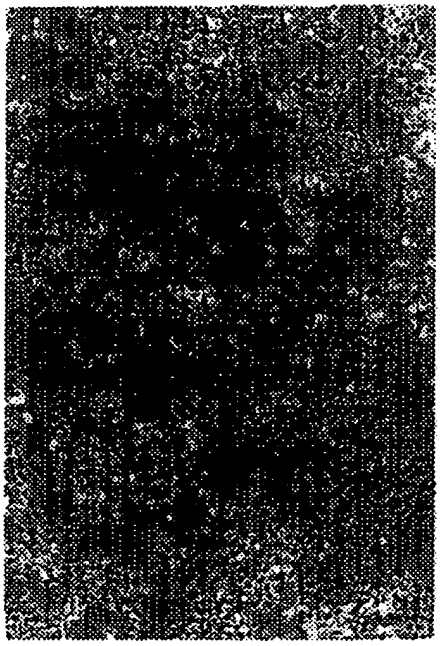

M U H B E C M (kb)
23
9.0
6.0
2.0

FIG.2C



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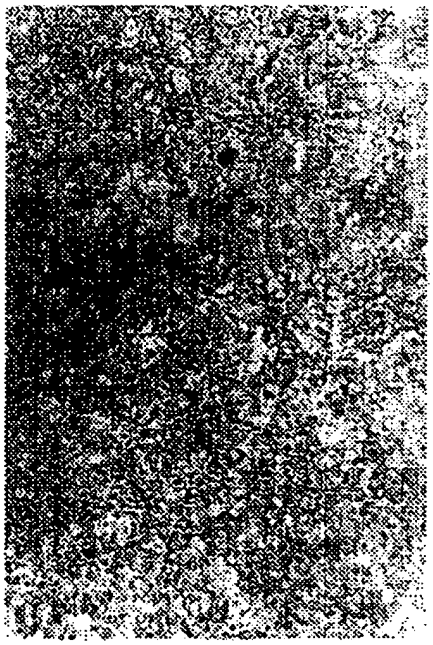

FIG.3B

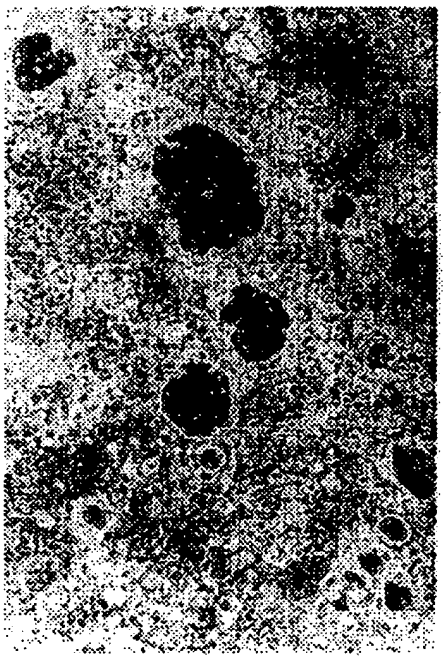

FIG.3C

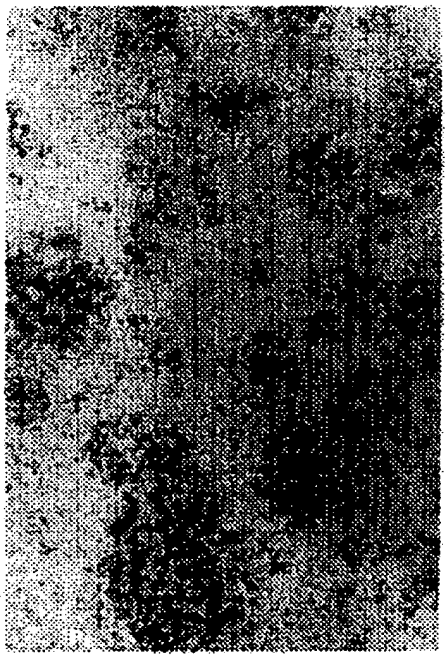

FIG.3D

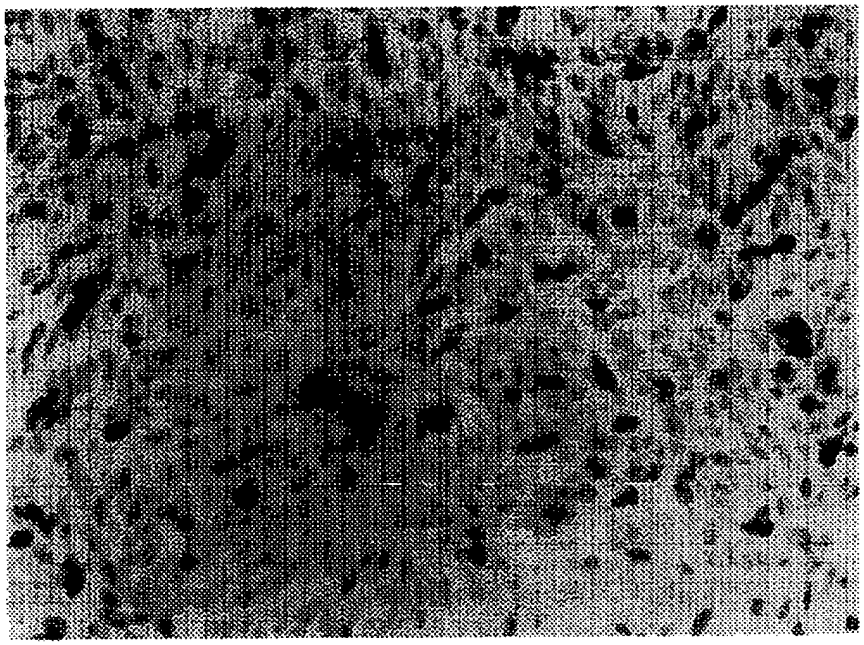

FIG.4A

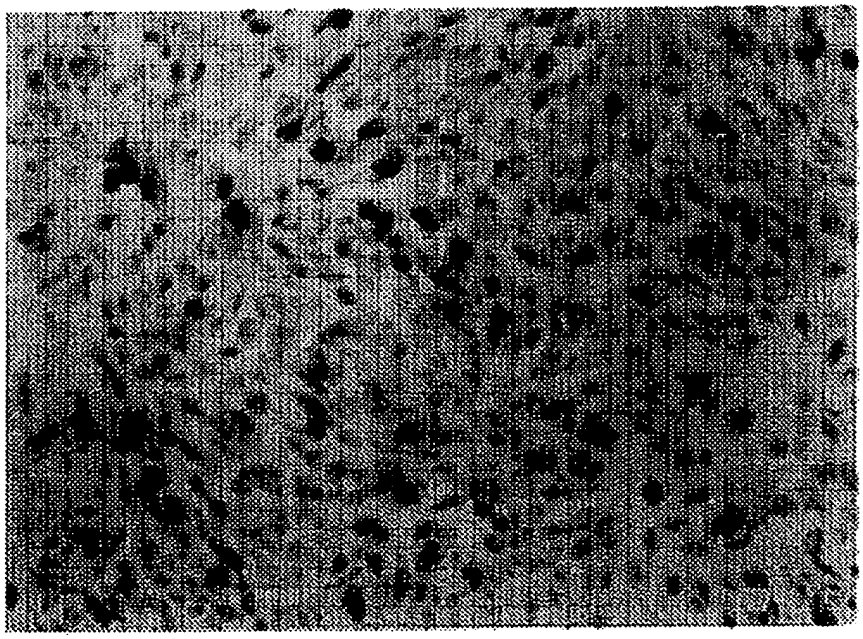

FIG.4B

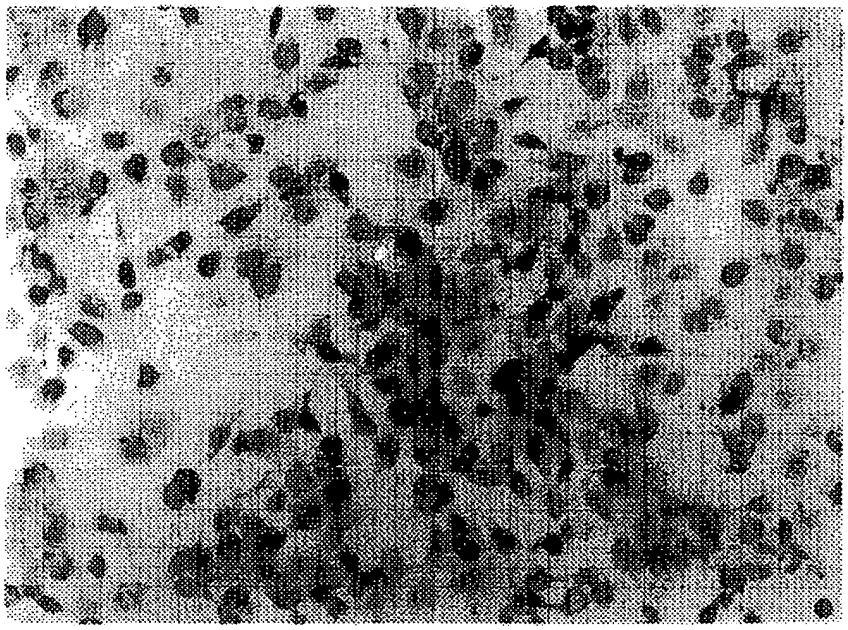

FIG.4C

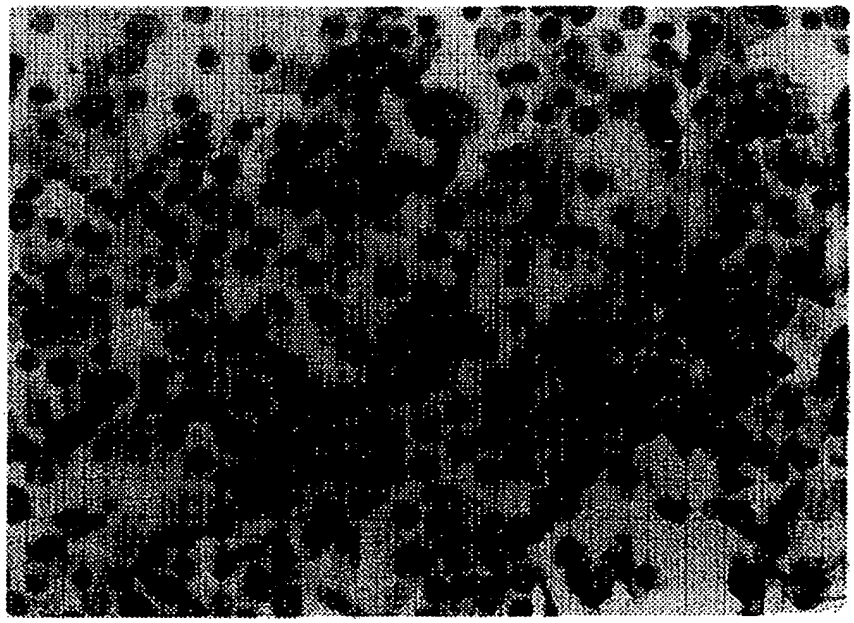

FIG.4D

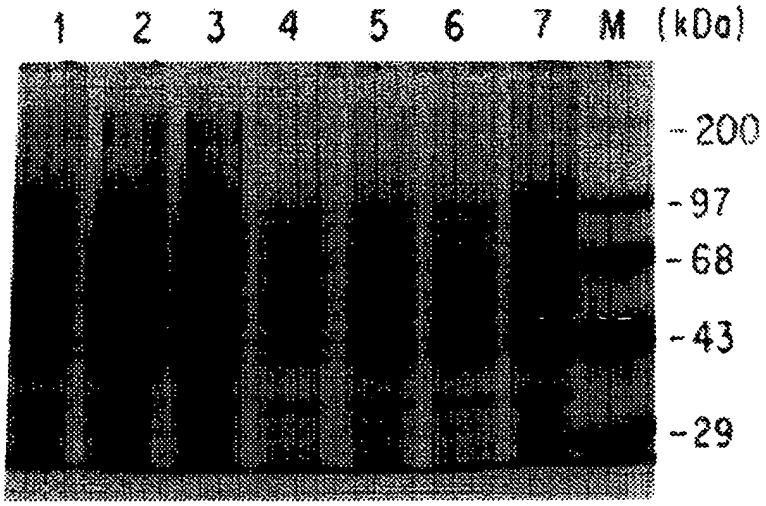

FIG.5A

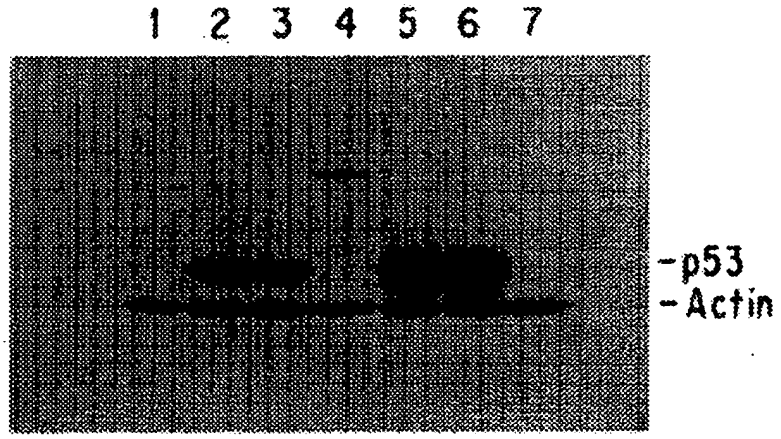

FIG.5B

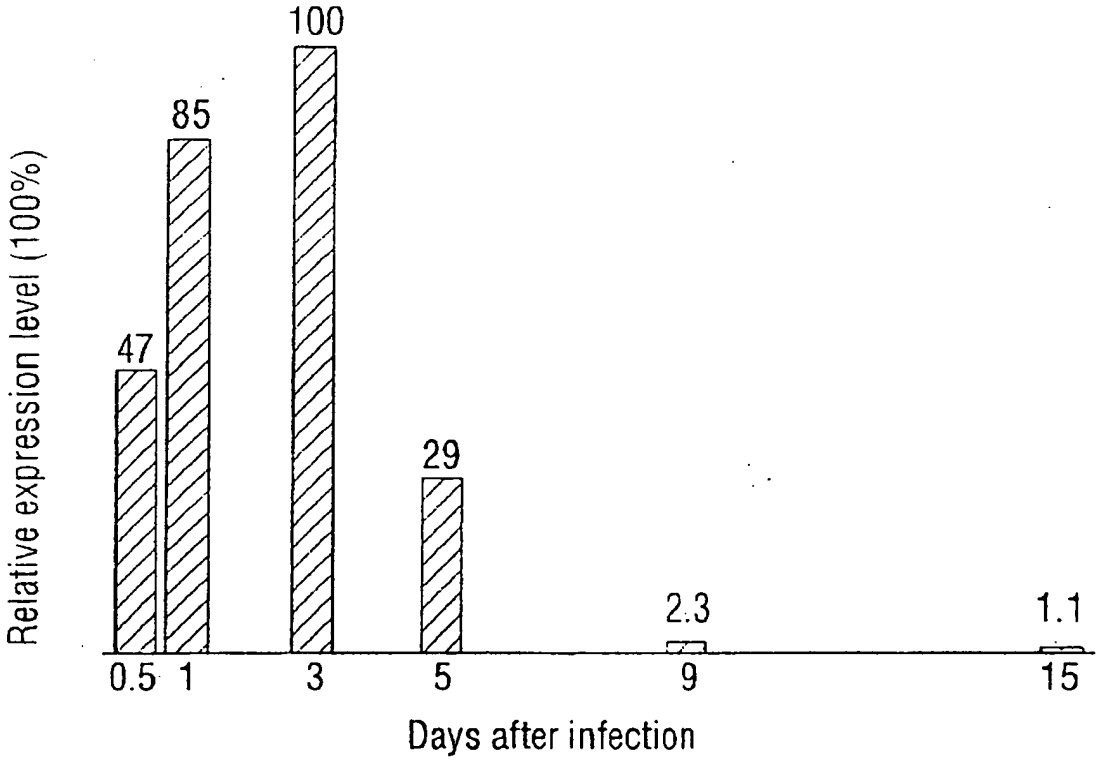
Relative expression level (100%)
47
85
100
29
2.3
1.1
0.5
1
3
5
9
15
Days after infection

FIG. 6A

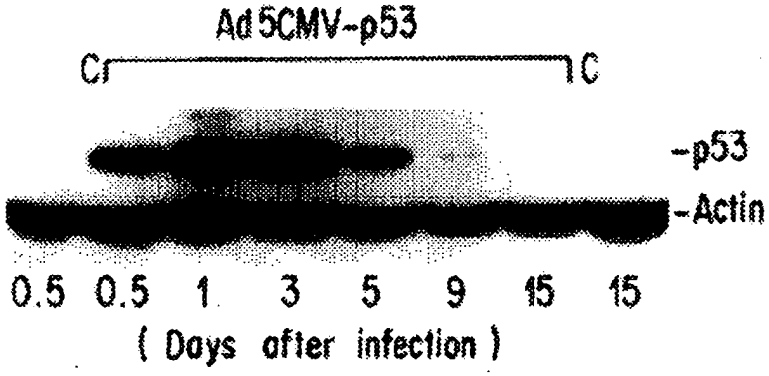
Ad5CMV-p53
Cr
C
-p53
-Actin
0.5 0.5 1 3 5 9 15 15
(Days after infection)

FIG.6B

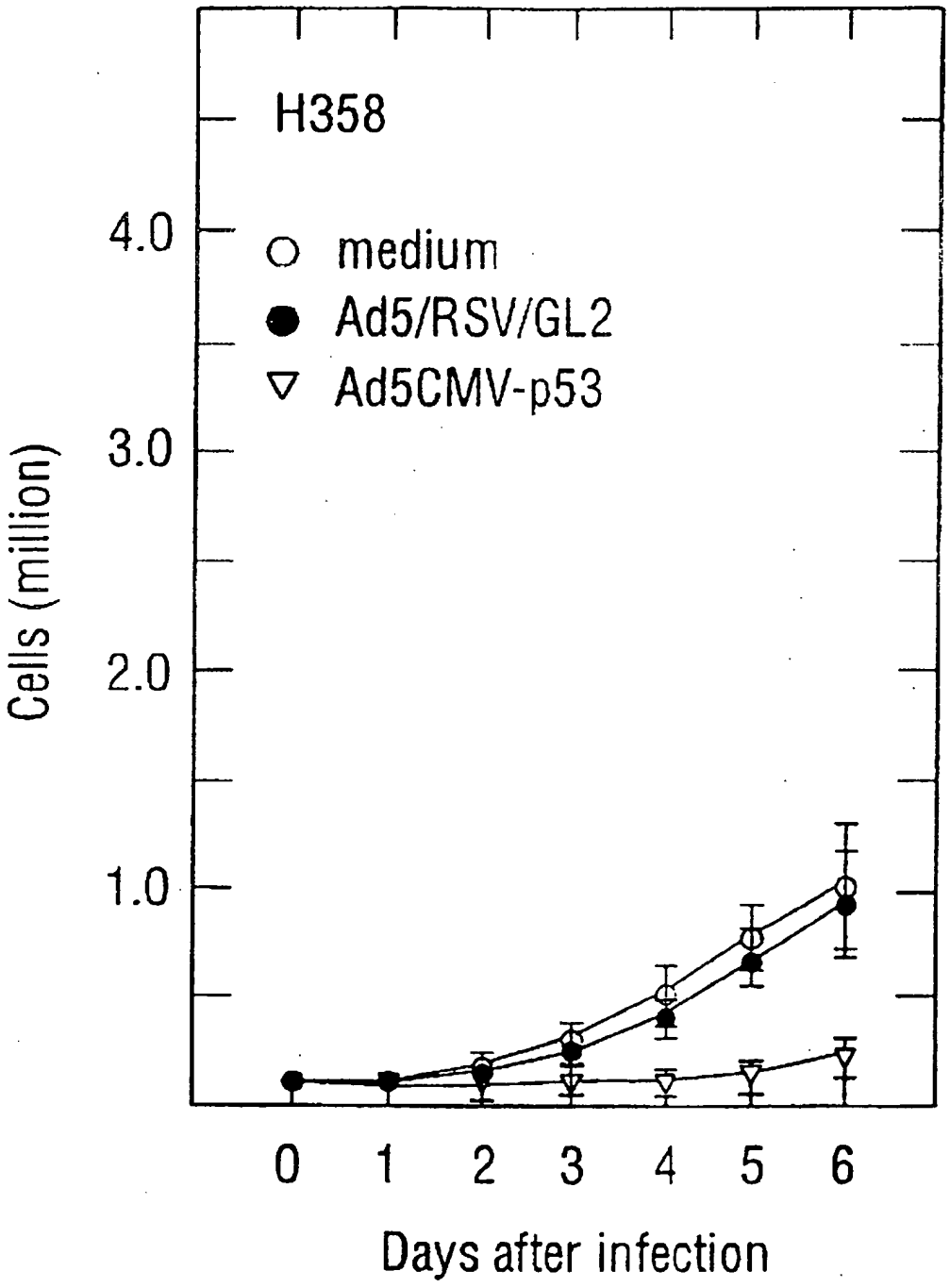
H358
medium
Ad5/RSV/GL2
Ad5CMV-p53
Cells (million)
4.0
3.0
2.0
1.0
0
1
2
3
4
5
6
Days after infection

FIG. 7A

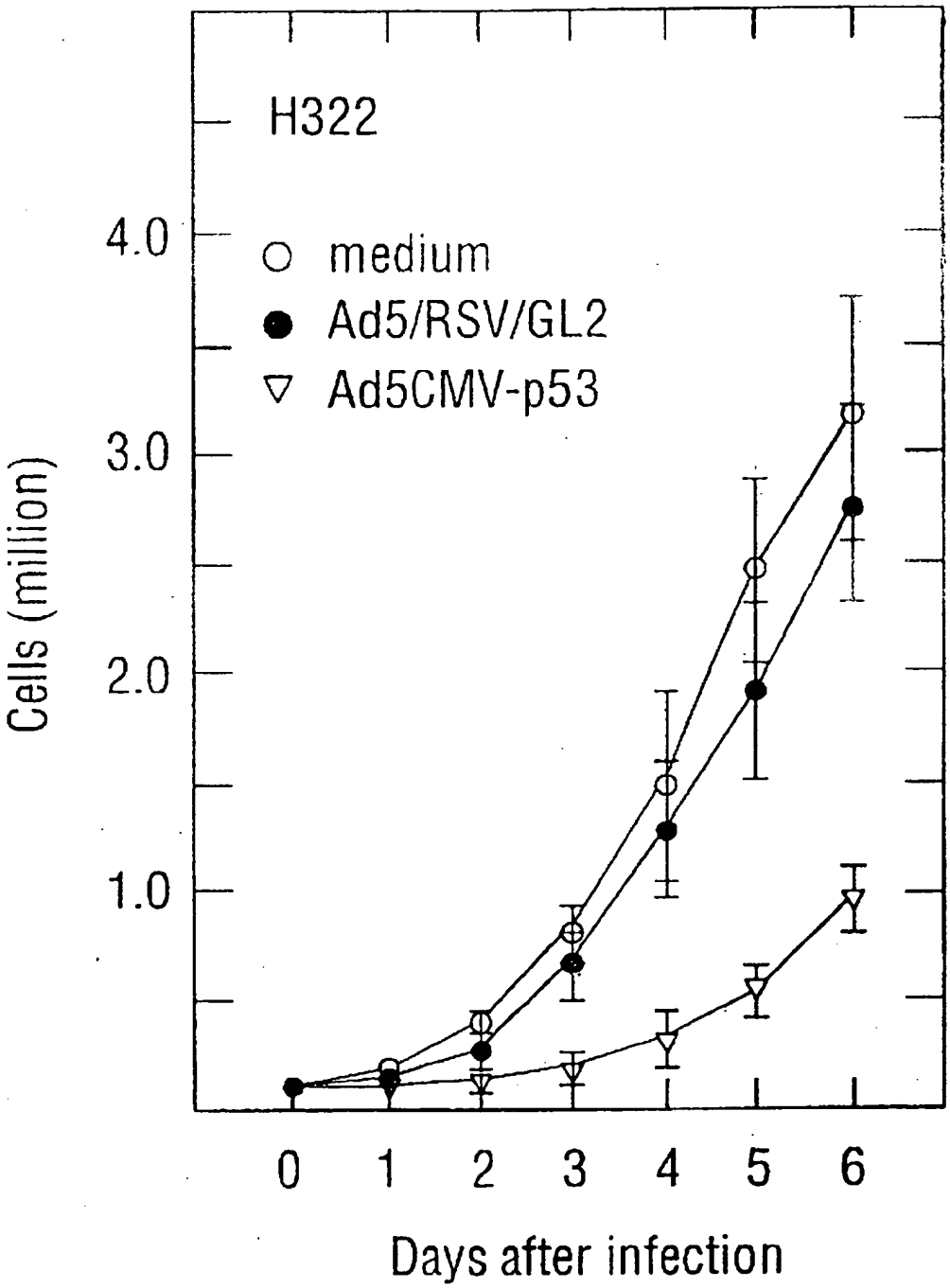
H322
medium
Ad5/RSV/GL2
Ad5CMV-p53
Cells (million)
4.0
3.0
2.0
1.0
0
1
2
3
4
5
6
Days after infection

FIG. 7B

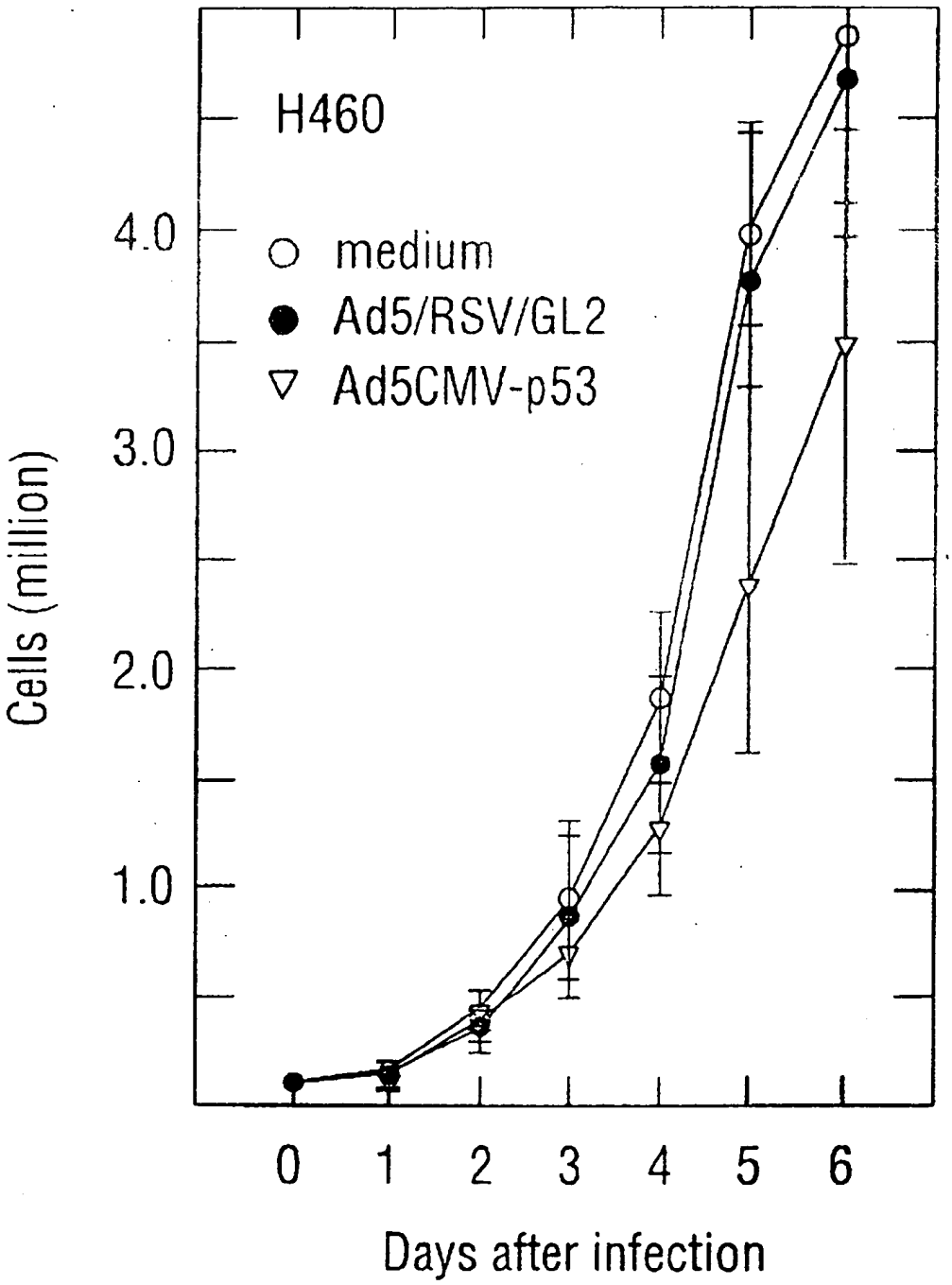
H460
medium
Ad5/RSV/GL2
Ad5CMV-p53
Cells (million)
4.0
3.0
2.0
1.0
0
1
2
3
4
5
6
Days after infection

FIG. 7C

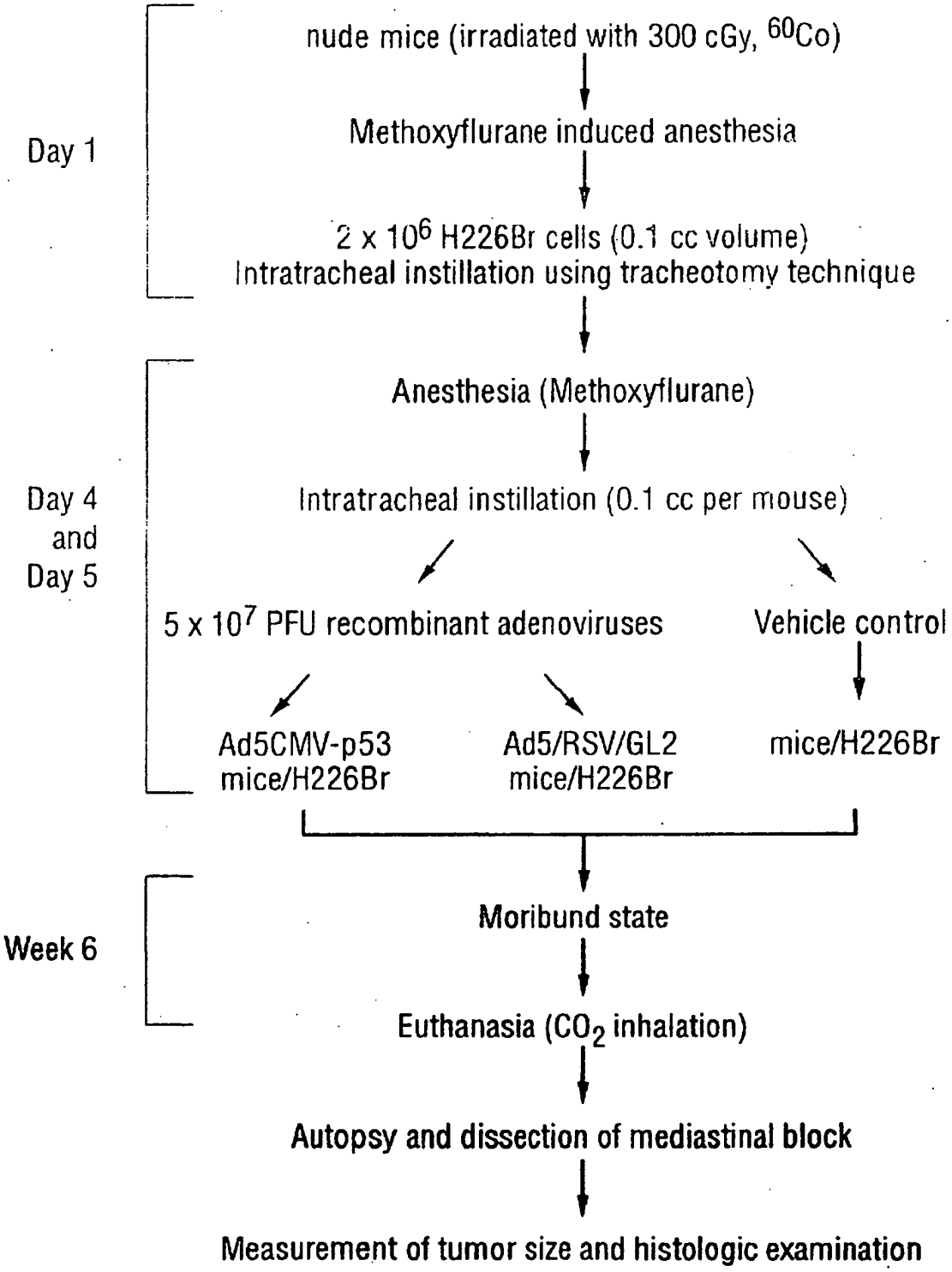
Day 1
nude mice (irradiated with 300 cGy, ^{60}Co)
Methoxyflurane induced anesthesia
2×10^6 H226Br cells (0.1 cc volume)
Intratracheal instillation using tracheotomy technique
Day 4 and Day 5
Anesthesia (Methoxyflurane)
Intratracheal instillation (0.1 cc per mouse)
5×10^7 PFU recombinant adenoviruses
Vehicle control
Ad5CMV-p53 mice/H226Br
Ad5/RSV/GL2 mice/H226Br
mice/H226Br
Week 6
Moribund state
Euthanasia (CO_2 inhalation)
Autopsy and dissection of mediastinal block
Measurement of tumor size and histologic examination

FIG. 8

FIG.9A

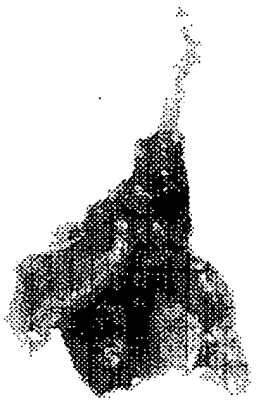

FIG.9B

FIG.9C

FIG.9D

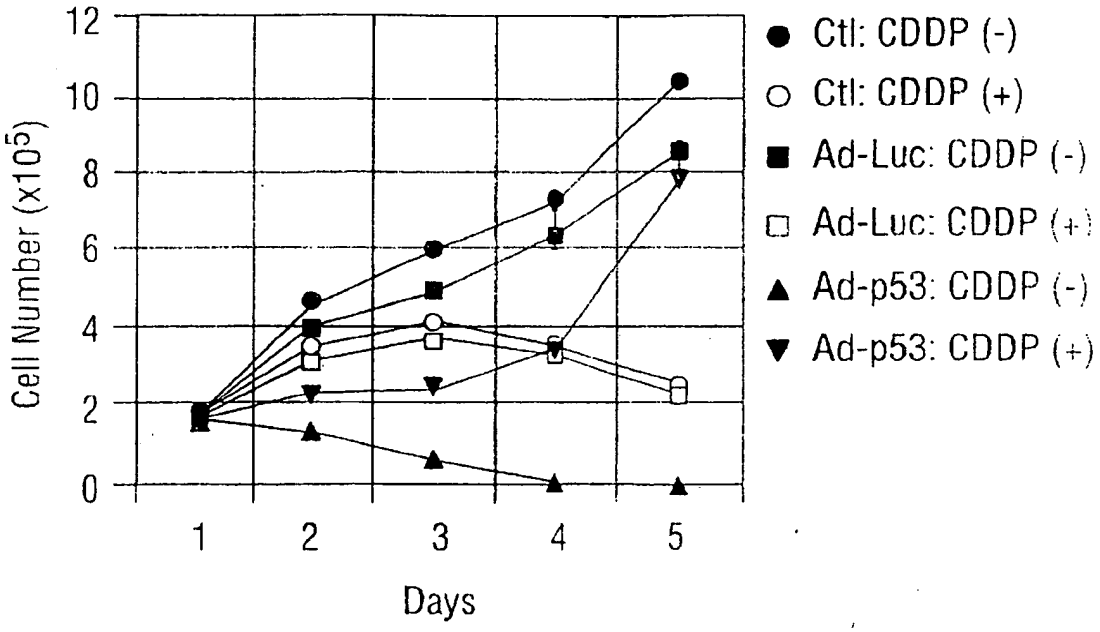

FIG. 10A

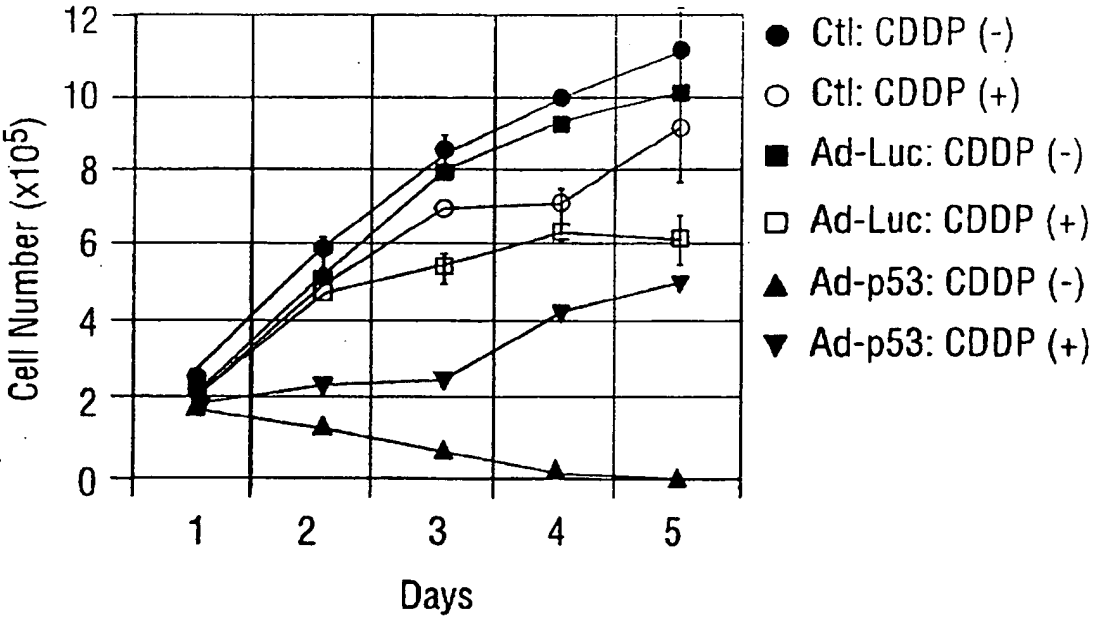

FIG. 10B

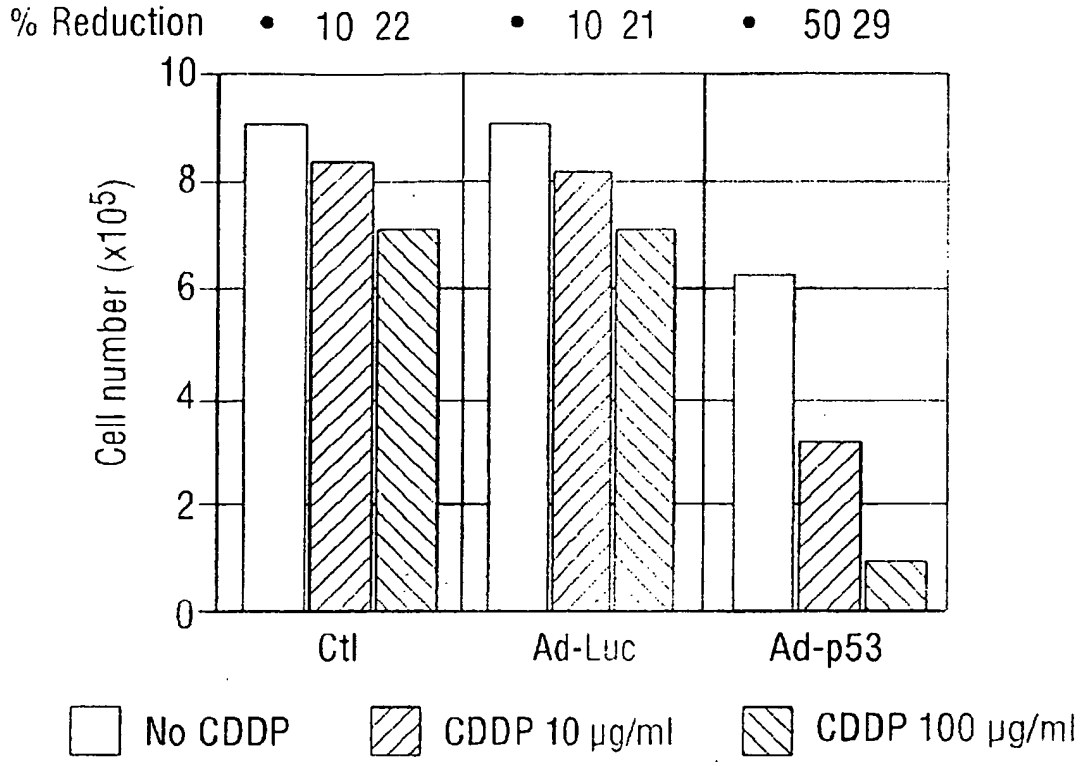

FIG. 10C

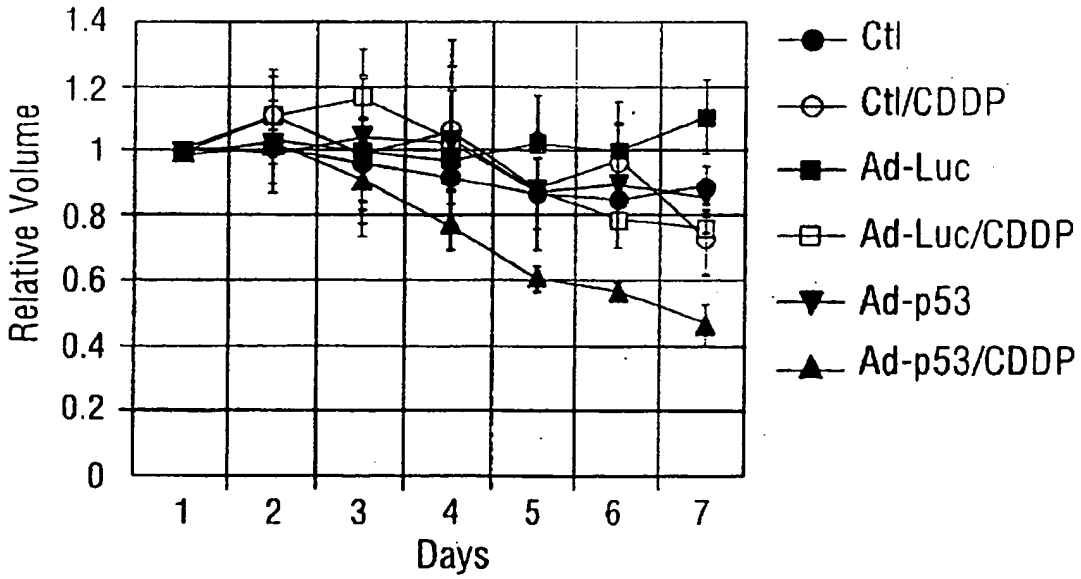

FIG. 12A

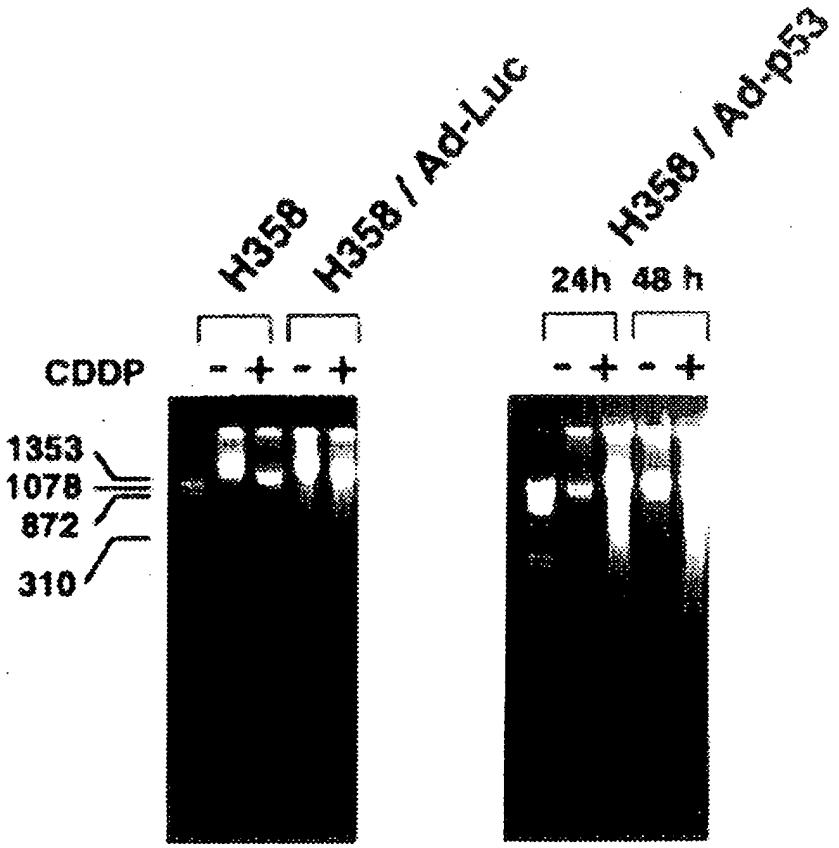

FIG. 11A

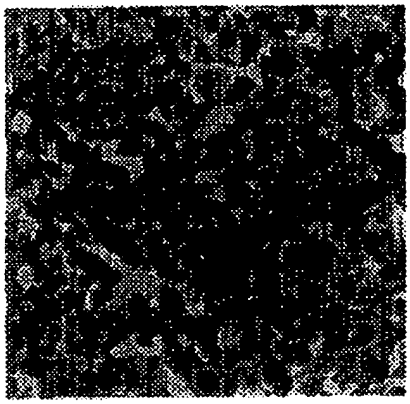

FIG.11B

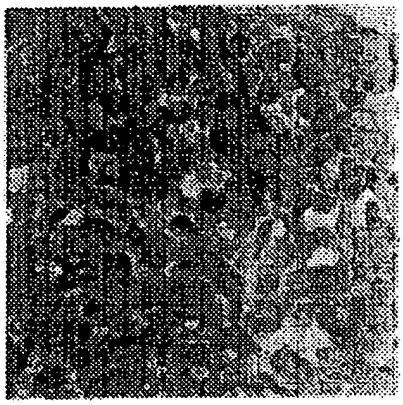

FIG.11C

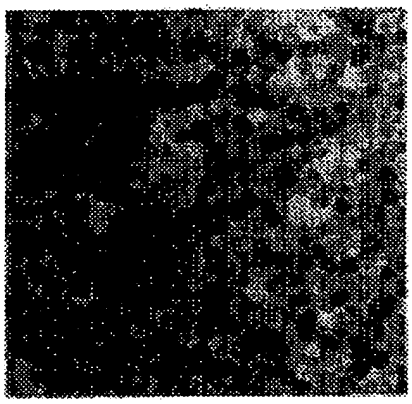

FIG.11D

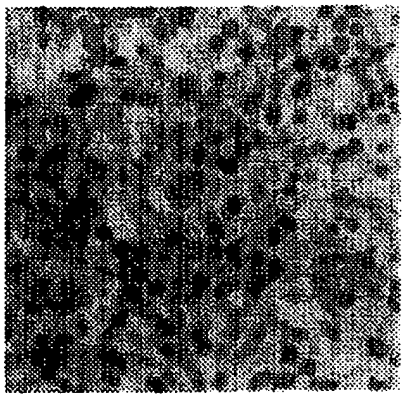

FIG.11E

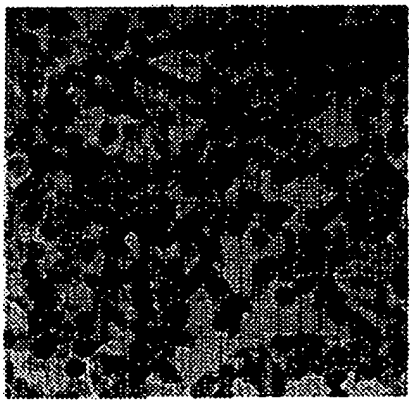

FIG.11F

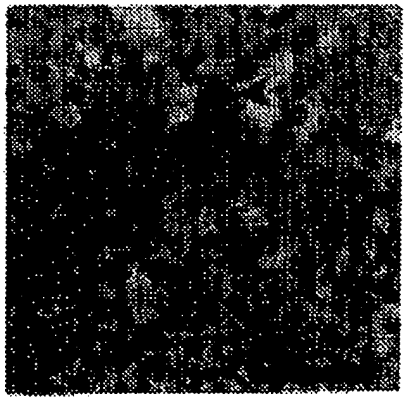

FIG.11G

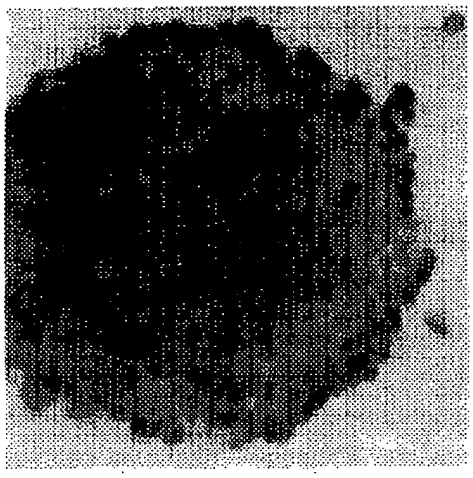

FIG.12B

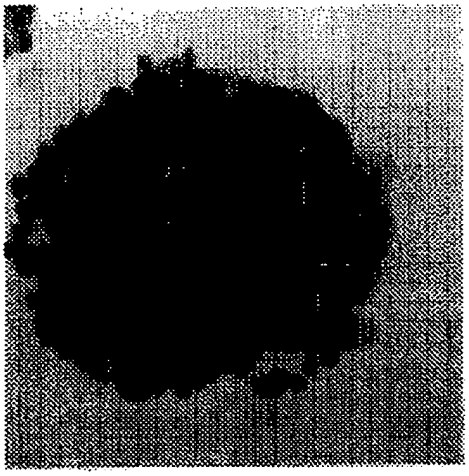

FIG.12C

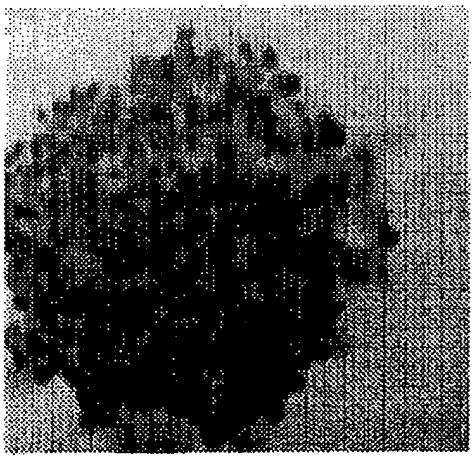

FIG.12D

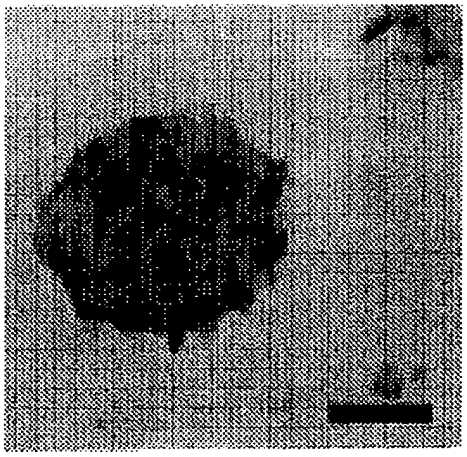

FIG.12E

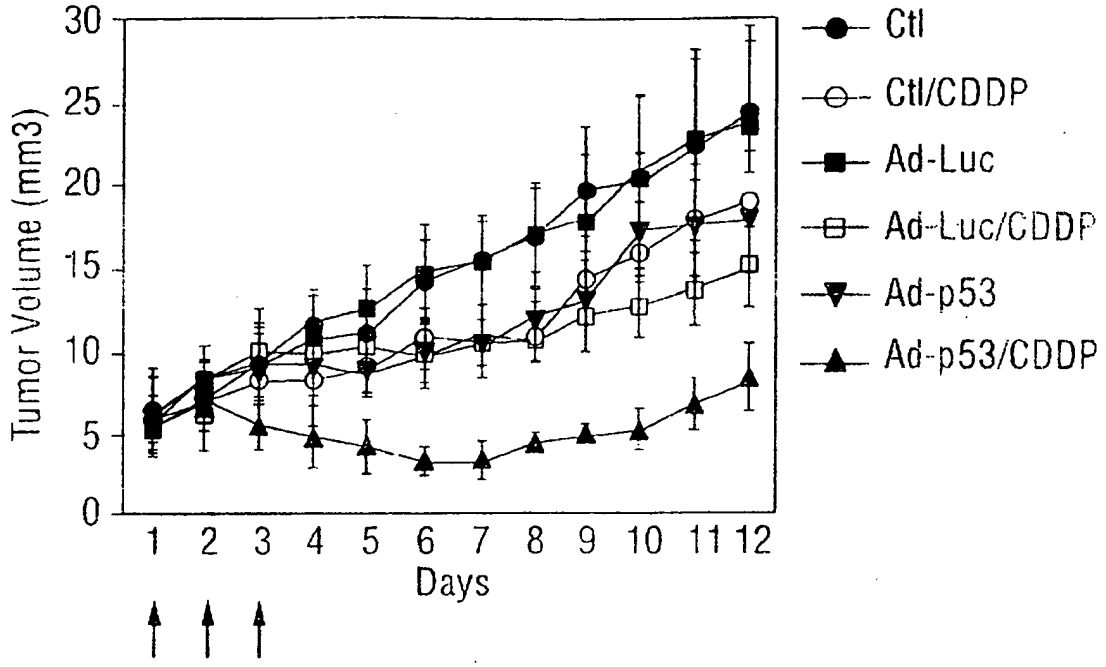

FIG. 13A-1

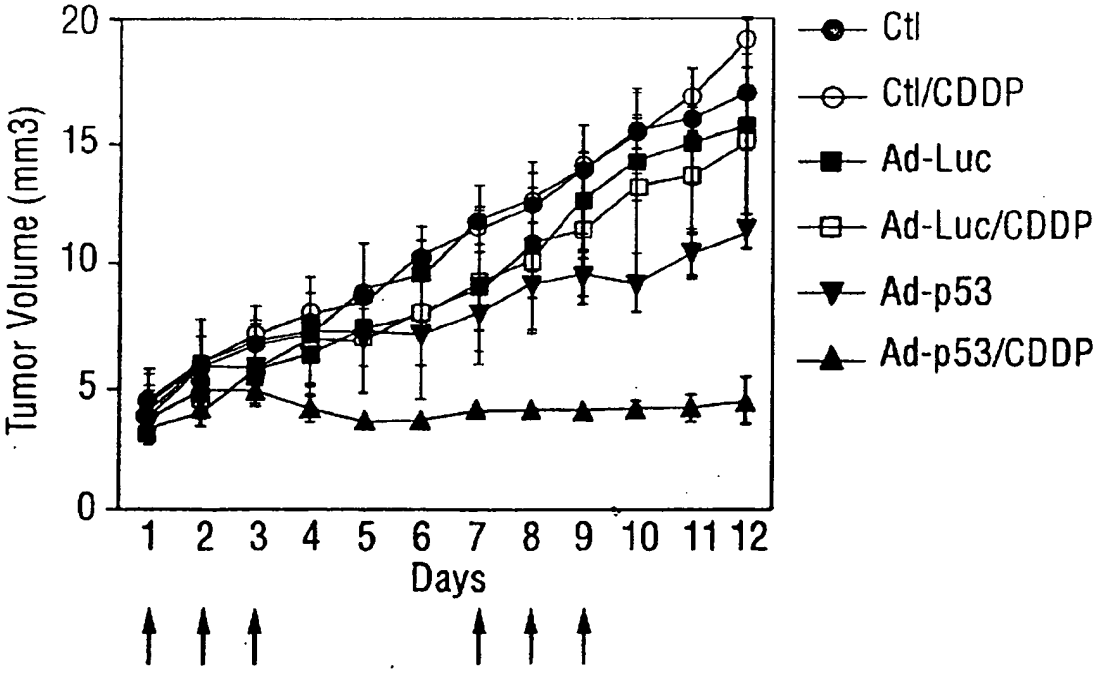

FIG. 13A-2

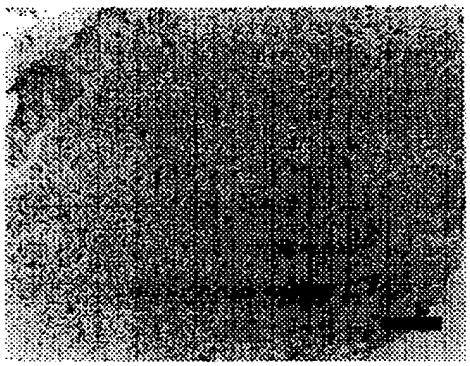

FIG.13B

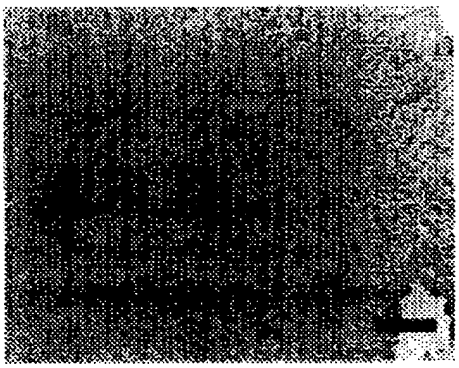

FIG.13C

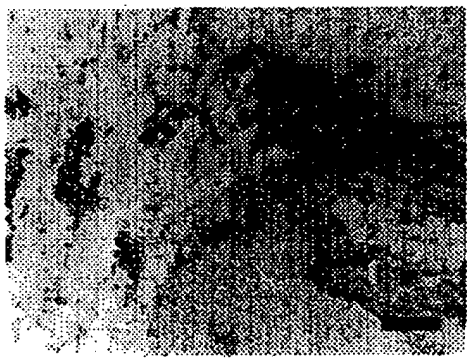

FIG.13D

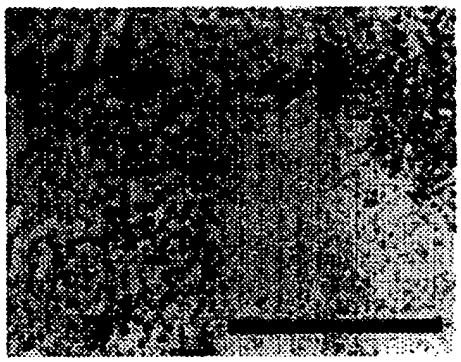

FIG.13E

METHODS AND COMPOSITIONS COMPRISING DNA DAMAGING AGENTS AND P53

The present application is a continuation-in-part of co-pending U.S. patent application Ser. No. 08/145,826, filed Oct. 29, 1993; which is a continuation-in-part of U.S. patent application Ser. No. 07/960,513, filed Oct. 13, 1992; which is a continuation-in-part of U.S. Ser. No. 07/665,538, filed Mar. 6, 1991 now abandoned; the entire text and figures of which disclosures are incorporated herein by reference without disclaimer.

The government owns rights in the present invention pursuant to NIH grants RO1 CA 45187 and CA 16672, and Training Grants CA 09611 and CA 45225.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates generally to the area of novel strategies for the improvement of chemotherapeutic intervention. In other aspects, the present invention provides novel methods and compositions that combine the potency of DNA damaging agents with the combined delivery of a tumor suppressor. The combination of DNA damaging factors with the heterologous expression of a tumor suppressor gene lead to a pronounced synergy over and above the actions of the individual components.

2. Description of Related Art

Current treatment methods for cancer, including radiation therapy, surgery, and chemotherapy, are known to have limited effectiveness. Lung cancer alone kills more than 140,000 people annually in the United States. Recently, age-adjusted mortality from lung cancer has surpassed that from breast cancer in women. Although implementation of smoking-reduction programs has decreased the prevalence of smoking, lung cancer mortality rates will remain high well into the 21st century. The rational development of new therapies for lung cancer will depend on an understanding of the biology of lung cancer at the molecular level.

It is now well established that a variety of cancers are caused, at least in part, by genetic abnormalities that result in either the over expression of one or more genes, or the expression of an abnormal or mutant gene or genes. For example, in many cases, the expression of oncogenes is known to result in the development of cancer. "Oncogenes" are genetically altered genes whose mutated expression product somehow disrupts normal cellular function or control (Spandidos et al., 1989).

Most oncogenes studied to date have been found to be "activated" as the result of a mutation, often a point mutation, in the coding region of a normal cellular gene, i.e., a "proto-oncogene", that results in amino acid substitutions in the expressed protein product. This altered expression product exhibits an abnormal biological function that takes part in the neoplastic process (Travali et al., 1990). The underlying mutations can arise by various means, such as by chemical mutagenesis or ionizing radiation. A number of oncogenes and oncogene families, including ras, myc, neu, raf, erb, src, fms, jun and abl, have now been identified and characterized to varying degrees (Travali et al., 1990; Bishop, 1987).

During normal cell growth, it is thought that growth-promoting proto-oncogenes are counterbalanced by growth-constraining tumor suppressor genes. Several factors may contribute to an imbalance in these two forces, leading to the neoplastic state. One such factor is mutations in tumor suppressor genes (Weinberg, 1991).

An important tumor suppressor gene is the gene encoding the cellular protein, p53, which is a 53 kD nuclear phosphoprotein that controls cell proliferation. Mutations to the p53 gene and allele loss on chromosome 17p, where this gene is located, are among the most frequent alterations identified in human malignancies. The p53 protein is highly conserved through evolution and is expressed in most normal tissues. Wild-type p53 has been shown to be involved in control of the cell cycle (Mercer, 1992), transcriptional regulation (Fields et al., 1990, and Mietz et al., 1992), DNA replication (Wilcock and Lane, 1991, and Bargonetti et al., 1991), and induction of apoptosis (Yonish-Rouach et al., 1991, and, Shaw et al., 1992).

Various mutant p53 alleles are known in which a single base substitution results in the synthesis of proteins that have quite different growth regulatory properties and, ultimately, lead to malignancies (Hollstein et al., 1991). In fact, the p53 gene has been found to be the most frequently mutated gene in common human cancers (Hollstein et al., 1991; Weinberg, 1991), and is particularly associated with those cancers linked to cigarette smoke (Hollstein et al., 1991; Zakut-Houri et al., 1985). The overexpression of p53 in breast tumors has also been documented (Casey et al., 1991).

One of the most challenging aspects of gene therapy for cancer relates to utilization of tumor suppressor genes, such as p53. It has been reported that transfection of wild-type p53 into certain types of breast and lung cancer cells can restore growth suppression control in cell lines (Casey et al., 1991; Takahasi et al., 1992). Although DNA transfection is not a viable means for introducing DNA into patients' cells, these results serve to demonstrate that supplying wild type p53 to cancer cells having a mutated p53 gene may be an effective treatment method if an improved means for delivering the p53 gene could be developed.

Gene delivery systems applicable to gene therapy for tumor suppression are currently being investigated and developed. Virus-based gene transfer vehicles are of particular interest because of the efficiency of viruses in infecting actual living cells, a process in which the viral genetic material itself is transferred. Some progress has been made in this regard as, for example, in the generation of retroviral vectors engineered to deliver a variety of genes. However, major problems are associated with using retroviral vectors for gene therapy since their infectivity depends on the availability of retroviral receptors on the target cells, they are difficult to concentrate and purify, and they only integrate efficiently into replicating cells.

Tumor cell resistance to chemotherapeutic drugs represents a major problem in clinical oncology. NSCLC accounts for at least 80% of the cases of lung cancer; patients with NSCLC are, however, generally unresponsive to chemotherapy (Doyle, 1993). One goal of current cancer research is to find ways to improve the efficacy of gene replacement therapy for cancer by investigating interaction between the gene product and chemotherapeutic drugs. The herpes simplex-thymidine kinase (HS-tK) gene, when delivered to brain tumors by a retroviral vector system, successfully induced susceptibility to the antiviral agent ganciclovir (Culver, et al., 1992). The HS-tK gene product is an exogenous viral enzyme, whereas the wt-p53 protein is expressed in normal tissues, suggesting that the modulation of chemoresistance by alterations in wt-p53 expression might be an alternative approach using a pathway mediated by an endogenous genetic program.

An adenovirus system has potential advantages for gene delivery in vivo, such as ease of producing high titer virus.

high infection efficiency, and infectivity for many types of cells. The stability and duration of expression of the introduced gene are still controversial, however. The increase in p53 levels in cells that are sensitive to chemotherapeutic drugs can occur within 6 hours after DNA-damaging stimuli (Fritsche, et al., 1993, Zhan, et al., 1993), although increased p53 DNA binding activity can be reversed over the course of 4 hours if the stimulus is removed (Tishler, et al., 1993). Therefore, a high level of p53 expression can be maintained even after cessation of drug exposure. The expression of wt-p53 protein by Ad-p53 peaks at postinfection day 3 (14-fold greater than endogenous wild type) and decreases to a low level by day 9 (Zhang, et al., 1993). This suggests that a transiently high level of wt-p53 expression is sufficient to initiate the cytotoxic program in the cancer cell.

p53 has an important role as a determinant of chemosensitivity in human lung cancer cells. A variety of treatment protocols, including surgery, chemotherapy, and radiotherapy, have been tried for human NSCLC, but the long-term survival rate remains unsatisfactory. What is needed is a combination therapy that is used alone or as an effective adjuvant treatment to prevent local recurrence following primary tumor resection or as a treatment that could be given by intralesional injections in drug-resistant primary, metastatic, or locally recurrent lung cancer. Compositions and methods are also needed to developed, explore and improve clinical applicability of novel compositions for the treatment of cancer. Furthermore these methods and compositions must prove their value in an in vivo setting.

SUMMARY OF THE INVENTION

The present invention addresses the need for improved therapeutic preparations for use in killing cells by combining the effects of a tumor suppressor gene or protein and a DNA damaging agent or factor. The present invention also provides compositions and methods, including those that use viral mediated gene transfer, to promote expression of a wild-type tumor suppressor gene, such as p53, in target cells and to deliver an agent or factor that induces DNA damage. The inventors surprisingly found that using the compositions disclosed herein, they were able to induce programmed cell death, also known as apoptosis, in a very significant number of target cells.

Using the present invention the inventors have demonstrated a remarkable effect in controlling cell growth and in particular, tumor cell growth. Tumor cell formation and growth, also known as "transformation", describes the formation and proliferation of cells that have lost their ability to control cellular division, that is, they are cancerous. It is envisioned that a number of different types of transformed cells are potential targets for the methods and compositions of the present invention, such as: sarcomas, melanomas, lymphomas, and a wide variety of solid tumors and the like. Although any tissue having malignant cell growth may be a target, lung and breast tissue are preferred targets. The present inventors disclose herein that a p53-expressing recombinant delivery vector was able to markedly reduce the growth rate of cells when used in conjunction with a DNA damaging agent.

The invention provides, in certain embodiments, methods and compositions for killing a cell or cells, such as a malignant cell or cells, by contacting or exposing a cell or population of cells with a p53 protein or gene and ne or more DNA damaging agents in a combined amount effective to kill the cell(s). Cells that may be killed using the invention include, e.g., undesirable but benign cells, such as benign prostate hyperplasia cells or over-active thyroid cells; cells relating to autoimmune diseases, such as B cells that produce antibodies involved in arthritis, lupus, myasthenia gravis, squamous metaplasia, dysplasia and the like. Although generally applicable to killing all undesirable cells, the invention has a particular utility in killing malignant cells. "Malignant cells" are defined as cells that have lost the ability to control the cell division cycle, as leads to a "transformed" or "cancerous" phenotype.

To kill cells, such as malignant or metastatic cells, using the methods and compositions of the present invention, one would generally contact a "target" cell with a p53 protein or gene and at least one DNA damaging agent in a combined amount effective to kill the cell. This process may involve contacting the cells with the p53 protein or gene and the DNA damaging agent(s) or factor(s) at the same time. This may be achieved by contacting the cell with a single composition or pharmacological formulation that includes both agents, or by contacting the cell with two distinct compositions or formulations, at the same time, wherein one composition includes the p53 protein or gene and the other includes the DNA damaging agent.

Naturally, it is also envisioned that the target cell may be first exposed to the DNA damaging agent(s) and then contacted with a p53 protein or gene, or vice versa. However, in embodiments where the DNA damaging factor and p53 are applied separately to the cell, one would generally ensure that a significant period of time did not expire between the time of each delivery, such that the DNA damaging agent and p53 would still be able to exert an advantageously combined effect on the cell. In such instances, it is contemplated that one would contact the cell with both agents within about 12–24 hours of each other, and more preferably within about 6–12 hours of each other, with a delay time of only about 12 hours being most preferred.

The terms "contacted" and "exposed", when applied to a cell, are used herein to describe the process by which a tumor suppressor gene or protein, such as p53, and a DNA damaging agent or factor are delivered to a target cell or are placed in direct juxtaposition with the target cell. To achieve cell killing, both agents are delivered to a cell in a combined amount effective to kill the cell, i.e., to induce programmed cell death or apoptosis. The terms, "killing", "programmed cell death" and "apoptosis" are used interchangeably in the present text to describe a series of intracellular events that lead to target cell death. The process of cell death involves the activation of intracellular proteases and nucleases that lead to, for example, cell nucleus involution and nuclear DNA fragmentation. An understanding of the precise mechanisms by which various intracellular molecules interact to achieve cell death is not necessary for practicing the present invention.

DNA damaging agents or factors are defined herein as any chemical compound or treatment method that induces DNA damage when applied to a cell. Such agents and factors include radiation and waves that induce DNA damage, such as, γ-irradiation, X-rays, UV-irradiation, microwaves, electronic emissions, and the like. A variety of chemical compounds, also described as "chemotherapeutic agents", function to induce DNA damage, all of which are intended to be of use in the combined treatment methods disclosed herein. Chemotherapeutic agents contemplated to be of use, include, e.g., adriamycin, 5-fluorouracil (5FU), etoposide (VP-16), camptothecin, actinomycin-D, mitomycin C, cisplatin (CDDP), and even hydrogen peroxide. The invention also encompasses the use of a combination of one or more DNA damaging agents, whether radiation-based or actual

compounds, such as the use of X-rays with cisplatin or the use of cisplatin with etoposide. In certain embodiments, the use of cisplatin in combination with a p53 protein or gene is particularly preferred as this compound.

Any method may also be used to contact a cell with a p53 protein, so long as the method results in increased levels of functional p53 protein within the cell. This includes both the direct delivery of a p53 protein to the cell and the delivery of a gene or DNA segment that encodes p53, which gene will direct the expression and production of p53 within the cell. In that protein delivery is subject to such drawbacks as protein degradation and low cellular uptake, it is contemplated that the use of a recombinant vector that expresses a p53 protein will provide particular advantages.

A wide variety of recombinant plasmids and vectors may be engineered to expresses a p53 protein and so used to deliver p53 to a cell. These include, for example, the use of naked DNA and p53 plasmids to directly transfer genetic material into a cell (Wolfe et al., 1990); formulations of p53-encoding DNA trapped in liposomes (Ledley et al., 1987) or in proteoliposomes that contain viral envelope receptor proteins (Nicolau et al., 1983); and p53-encoding DNA coupled to a polylysine-glycoprotein carrier complex.

The use of recombinant viruses engineered to express p53 is also envisioned. A variety of viral vectors, such as retroviral vectors, herpes simplex virus (U.S. Pat. No. 5,288,641, incorporated herein by reference), cytomegalovirus, and the like may be employed, as described by Miller (Miller, 1992); as may recombinant adeno-associated virus (AAV vectors), such as those described by U.S. Pat. No. 5,139,941, incorporated herein by reference; and, particularly, recombinant adenoviral vectors. Techniques for preparing replication-defective infective viruses are well known in the art, as exemplified by Ghosh-Choudhury & Graham (1987); McGrory et al. (1988); and Gluzman et al. (1982), each incorporated herein by reference.

To kill a cell in accordance with the present invention, one would generally contact the cell with a p53 protein or gene and a DNA damaging agent in a combined amount effective to kill the cell. The term "in a combined amount effective to kill the cell" means that the amount of p53 and DNA damaging agents are sufficient so that, when combined within the cell, the cell is induced to undergo apoptosis. Although not required in all embodiments, the combined effective amount of p53 and DNA damaging agent will preferably be an amount that induces significantly more cell death than the use of either element alone, and most preferably, the combined effective amount will be an amount that induces synergistic cell death in comparison to the effects observed using either element alone.

A number of in vitro parameters may be used to determine the effect produced by the compositions and methods of the present invention. These parameters include, for example, the observation of net cell numbers before and after exposure to the compositions described herein, as well as the size of multicellular tumor spheroids formed, such as those colonies formed in tissue culture. In vitro cell killing is particularly shown in Example 7 of the present disclosure. Alternatively, one may measure parameters that are indicative of a cell that is undergoing programmed cell death, such as, the fragmentation of cellular genomic DNA into nucleosome size fragments, generally identified by separating the fragments by agarose gel electrophoresis, staining the DNA, and comparing the DNA to a DNA size ladder. Nucleosome size fragments are identified as a progressive steps or ladders of monomers and multimers having a base unit of about 200 basepairs.

Similarly, a "therapeutically effective amount" is an amount of a p53 protein or gene and DNA damaging agent that, when administered to an animal in combination, is effective to kill cells within the animal. This is particularly evidenced by the killing of cancer cells, such as lung, breast or colon cancer cells, within an animal or human subject that has a tumor. "Therapeutically effective combinations" are thus generally combined amounts of p53 and DNA damaging agents that function to kill more cells than either element alone, and preferably, combined amounts that bring about a synergistic reduction in tumor burden.

Studying certain in vivo and ex vivo parameters of cell death are therefore also effective means by which to assess the effectiveness of the composition and methods of the invention. For example, observing effects on the inhibition of tumorigenicity, as measured by TdT expression of frozen tissue sections or by using other staining methods and target antigens, as known to skilled pathologists. Naturally, other means of determining tumor mass, growth, and viability may also be used to assess the killing of target cells. In particular, one may assess the effects in various animal model systems of cancer, including those in which human cancer cells are localized within the animal. Animal models of cancer, unlike those of AIDS, are known to be highly predictive of human treatment regimens (Roth et al., editors (1989)). One exemplary embodiment of a predictive animal model is that in which human small-cell lung cancer cells (H358 cells) are grown subcutaneously. Using this system, the inventors have shown that p53-bearing adenovirus instilled intratumorally, along with the co-administration of a chemotherapeutic agent, gives rise to a surprisingly effective tumor reduction.

A particularly preferred method of delivering a p53 protein to a cell is to contact the cell with a recombinant adenovirus virion or particle that includes a recombinant adenoviral vector comprising a p53 expression region positioned under the control of a promoter capable of directing the expression of p53 in the given cell type.

The p53 expression region in the vector may comprise a genomic sequence, but for simplicity, it is contemplated that one will generally prefer to employ a p53 cDNA sequence as these are readily available in the art and more easily manipulated. In addition to comprising a p53 expression unit and a promoter region, the vector will also generally comprise a polyadenylation signal, such as an SV40 early gene, or protamine gene, polyadenylation signal, or the like.

In preferred embodiments, it is contemplated that one will desire to position the p53 expression region under the control of a strong constitutive promoter such as a CMV promoter, viral LTR, RSV, or SV40 promoter, or a promoter associated with genes that are expressed at high levels in mammalian cells such as elongation factor-1 or actin promoters. All such variants are envisioned to be useful with the present invention. Currently, a particularly preferred promoter is the cytomegalovirus (CMV) IE promoter.

The p53 gene or cDNA may be introduced into a recombinant adenovirus in accordance with the invention simply by inserting or adding the p53 coding sequence into a viral genome. However, the preferred adenoviruses will be replication defective viruses in which a viral gene essential for replication and/or packaging has been deleted from the adenoviral vector construct, allowing the p53 expression region to be introduced in its place. Any gene, whether essential (e.g., E1A, E1B, E2 and E4) or non-essential (e.g., E3) for replication, may be deleted and replaced with p53. Particularly preferred are those vectors and virions in which

the E1A and E1B regions of the adenovirus vector have been deleted and the p53 expression region introduced in the place, as exemplified by the genome structure of FIG. 1.

Techniques for preparing replication defective adenoviruses are well known in the art, as exemplified by Ghosh-Choudhury and Graham (1987); McGrory et al. (1988); and Gluzman et al., each incorporated herein by reference. It is also well known that various cell lines may be used to propagate recombinant adenoviruses, so long as they complement any replication defect which may be present. A preferred cell line is the human 293 cell line, but any other cell line that is permissive for replication, i.e., in the preferred case, which expresses E1A and E1B may be employed. Further, the cells can be propagated either on plastic dishes or in suspension culture, in order to obtain virus stocks thereof.

The invention is not limited to E1-lacking virus and E1-expressing cells alone. Indeed, other complementary combinations of viruses and host cells may be employed in connection with the present invention. Virus lacking functional E2 and E2-expressing cells may be used, as may virus lacking functional E4 and E4-expressing cells, and the like. Where a gene which is not essential for replication is deleted and replaced, such as, for example, the E3 gene, this defect will not need to be specifically complemented by the host cell.

Other than the requirement that the adenovirus vectors be engineered to express p53, the nature of the initial adenovirus is not believed to be crucial to the successful practice of the invention. The adenovirus may be of any of the 42 different known serotypes or subgroups A–F. Adenovirus type 5 of subgroup C is the preferred starting material in order to obtain the conditional replication-defective adenovirus vector for use in the method of the present invention. This is because Adenovirus type 5 is a human adenovirus about which there is significant amount of biochemical and genetic information known, and which has historically been used for most constructions employing adenovirus as a vector.

The methods and compositions of the present invention are equally suitable for killing a cell or cells both in vitro and in vivo. When the cells to be killed are located within an animal, e.g., lung, breast or colon cancer cells or other cells bearing a p53 mutation, both the p53 protein or gene and the DNA damaging agent will be administered to the animal in a pharmacologically acceptable form. The term "a pharmacologically acceptable form", as used herein, refers to both the form of any composition that may be administered to an animal, and also the form of contacting an animal with radiation, i.e., the manner in which an area of the animals body is irradiated, e.g., with γ-irradiation, X-rays, UV-irradiation, microwaves, electronic emissions, and the like. The use of DNA damaging radiation and waves is known to those skilled in the art of irradiation therapy.

The present invention also provides advantageous methods for treating cancer that, generally, comprise administering to an animal or human patient with cancer a therapeutically effective combination of a p53 protein or gene and a DNA damaging agent. This may be achieved using a recombinant virus, particularly an adenovirus, that carries a vector capable of expressing p53 in the cells of the tumor. The p53 gene delivering composition would generally be administered to the animal, often in close contact to the tumor, in the form of a pharmaceutically acceptable composition. Direct intralesional injection of a therapeutically effective amount of a p53 gene, such as housed within a recombinant virus, into a tumor site is one preferred method. However, other parenteral routes of administration, such as intravenous, percutaneous, endoscopic, or subcutaneous injection are also contemplated.

In treating cancer according to the invention one would contact the tumor cells with a DNA damaging agent in addition to the p53 protein or gene. This may be achieved by irradiating the localized tumor site with DNA damaging radiation such as X-rays, UV-light, γ-rays or even microwaves. Alternatively, the tumor cells may be contacted with the DNA damaging agent by administering to the animal a therapeutically effective amount of a pharmaceutical composition comprising a DNA damaging compound, such as, adriamycin, 5-fluorouracil, etoposide, camptothecin, actinomycin-D, mitomycin C, or more preferably, cisplatin. The DNA damaging agent may be prepared and used as a combined therapeutic composition, or kit, by combining it with a p53 protein, gene or gene delivery system, as described above.

The surprising success of the present invention is evidenced by the finding that using Ad5CMV-p53 virus in combination with cisplatin yielded profound results in studies using a nude mouse model. The combined virus-DNA damage therapy regimen significantly inhibited the tumorigenicity of H358 cells, a cell that normally produces a significant tumor mass. The tumorigenicity of the lung cancer cells was inhibited through the treatment by Ad5CMV-p53, but not by the control virus expressing luciferase, indicating that the p53 protein in combination with a DNA-damaging agent has great therapeutic efficacy.

A number of methods for delivering chemotherapeutic formulations, including DNA expression constructs, into eukaryotic cells are known to those of skill in the art. In light of the present disclosure, the skilled artisan will be able to deliver both DNA damaging agents and p53 proteins or genes to cells in many different effective ways.

For in vivo delivery of DNA, the inventors envision the use of any gene delivery system, such as viral- and liposome-mediated transfection. As used herein, the term "transfection", is used to describe the targeted delivery of DNA to eukaryotic cells using delivery systems, such as, adenoviral, AAV, retroviral, or plasmid delivery gene transfer methods. The specificity of viral gene delivery may be selected to preferentially direct the gene to a particular target cell, such as by using viruses that are able to infect particular cell types. Naturally, different viral host ranges will dictate the virus chosen for gene transfer, as well as the likely tumor suppressor gene to be expressed for killing a given malignant cell type.

It is also envisioned that one may provide the DNA damaging chemotherapeutic agent through a variety of means, such as by using parenteral delivery methods such as intravenous and subcutaneous injection, and the like. Such methods are known to those of skill in the art of drug delivery, and are further described herein in the sections regarding pharmaceutical preparations and treatment.

For in vitro gene delivery, a variety of methods may be employed, such as, e.g., calcium phosphate- or dextran sulfate-mediated transfection; electroporation; glass projectile targeting; and the like. These methods are known to those of skill in the art, with the exact compositions and executi n being apparent in light of the present disclosure.

Other embodiments concern compositions, including pharmaceutical formulations, comprising a p53 protein or gene in combination with a DNA damaging agent, such as cisplatin. In such compositions, the p53 may be in the form

a DNA segment, recombinant vector or recombinant virus that is capable of expressing a p53 protein in an animal cell. These compositions, including those comprising a recombinant viral gene delivery system, such as an adenovirus particle, may be formulated for in vivo administration by dispersion in a pharmacologically acceptable solution or buffer. Preferred pharmacologically acceptable solutions include neutral saline solutions buffered with phosphate, lactate, Tris, and the like.

Of course, in using viral delivery systems, one will desire to purify the virion sufficiently to render it essentially free of undesirable contaminants, such as defective interfering viral particles or endotoxins and other pyrogens such that it will not cause any untoward reactions in the cell, animal or individual receiving the vector construct. A preferred means of purifying the vector involves the use of buoyant density gradients, such as cesium chloride gradient centrifugation.

Preferred pharmaceutical compositions of the invention are those that include, within a pharmacologically acceptable solution or buffer, a p53 protein, or more preferably a p53 gene, in combination with a chemotherapeutic DNA damaging agent. Exemplary chemotherapeutic agents are adriamycin, 5-fluorouracil, camptothecin, actinomycin-D, hydrogen peroxide, mitomycin C, cisplatin (CDDP), and etoposide (VP-16), with the use of cisplatin being particularly preferred.

Still further embodiments of the present invention are kits for use in killing cells, such as malignant cells, as may be formulated into therapeutic kits for use in cancer treatment. The kits of the invention will generally comprise, in suitable container means, a pharmaceutical formulation of a recombinant vector that is capable of expressing a p53 protein in an animal cell, and a pharmaceutical formulation of a DNA damaging agent. The recombinant vectors and DNA damaging agents may be present within a single container, or these components may be provided in distinct or separate container means. In a preferred embodiment, the recombinant vector will be a recombinant p53-expressing adenoviral vector present within an adenovirus particle and the DNA damaging agent will be cisplatin.

The components of the kit are preferably provided as a liquid solution, or as a dried powder. When the components are provided in a liquid solution, the liquid solution is an aqueous solution, with a sterile aqueous solution being particularly preferred. When reagents or components are provided as a dry powder, the powder can be reconstituted by the addition of a suitable solvent. It is envisioned that the solvent may also be provided in another container means.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1. Scheme for generation of recombinant p53 adenovirus. The p53 expression cassette was inserted between the Xba I and Cla I sites of pXCJL.1. The p53 expression vector (pEC53) and the recombinant plasmid pJM17 were cotransfected into 293 cells. The transfected cells were maintained in medium until the onset of the cytopathic effect. Identification of newly generated p53 recombinant adenoviruses (Ad5CMV-p53) by PCR analysis of the DNA using DNA templates prepared from the CPE supernatants treated with Proteinase K and phenol extraction.

FIG. 2A. Map used for the structural analysis of Ad5CMV-p53 DNA. A map of Ad5CMV-p53 genomic DNA, with the locations of the p53 expression cassette, the PCR primers, and the restriction sites. The genome size is about 35.4 kb, divided into 100 maps units (1 m.u.=0.35 kb). The p53 expression cassette replaced the E1 region (1.3–9.2 m.u.) of the Ad5 genome. Primer 1 is located in the first intron downstream of the human CMV major IE gene promoter. Primer 2 is located in SV40 early polyadenylation signal. Both of the primers, 15–20 bp away from the p53 cDNA insert at both ends, define a 1.40 kb PCR product. Primers 3 and 4 are located at 11 m.u. and 13.4 m.u. of Ads genome, respectively, which define a 0.86 kb viral-genome specific PCR product.

FIG. 2B. Agarose gel analysis of PCR products. Two pairs of primers that define 1.4-kb (p53) and 0.86-kb (Ad5) DNA fragments were used in each reaction. DNA templates used in each reaction were pEC53 plasmid (lane 1), Ad5/RSV/GL2 DNA (lane 2), no DNA (lane 3), and Ad5CMV-p53 DNA (lane 4). The lane labeled (M) corresponds to molecular weight markers.

FIG. 2C. Restriction mapping of Ad5CMV-p53 DNA. CsCl-gradient purified Ad5CMV-p53 DNA samples were digested with no enzyme (U), Hind III (H), Bam HI (B), Eco RI (E), and Cla I (C), respectively, and analyzed on 1% agarose gel. The lanes labeled (M) are molecular weight markers.

FIGS. 3A, 3B, 3C and 3D. Observation of cytopathic effects on 293 by recombinant adenovirus. FIGS. 3A, 3B, 3C and 3D are a series of phase contrast images (×400) of 293 cells. FIGS. 3A, 3B, 3C and 3D are four panels of a single page figure. FIG. 2A, before transfection; FIG. 3B, negative control on day 12 posttransfection; FIG. 3C, onset of CPE on day 12 posttransfection; FIG. 3D, completion of CPE on day 14 post-transfection.

FIGS. 4A, 4B, 4C, and 4D. Immunohistology of cells infected with recombinant adenoviruses. FIGS. 4A, 4B, 4C and 4D are a series of immunohistological images of H358 cells. FIGS. 4A, 4B, 4C and 4D are four panels of a single page figure. Infectivity of Ad5CMV-p53 in H358 cells. H358 cells were infected with Ad5CMV-p53 or Ad5/RSV/GL2 at 50 PFU/cell for 24 h. Medium alone was used as a mock infection. The infected cells were analyzed by immunostainings. FIG. 4A is a mock infection probed with anti-p53 antibody. FIG. 4B are cells infected with the Ad5/RSV/GL2 control and probed with anti-p53 antibody. FIG. 4C are Ad5CMV-p53 infected cells probed with an unrelated antibody (MOPC-21). FIG. 4D are cells Ad5CMV-p53 infection probed with anti-p53 antibody. The anti-p53 antibody used was Pab 1801, and the avidin-biotin method was used for staining.

FIG. 5A. Coomassie-blue stained SDS-PAGE gel comparing the relative level of expression of exogenous p53 in H358 cells. H358 cell samples that were infected with Ad5CMV-p53 or Ad5/RSV/GL2 at 30 PFU/cell were prepared 24 and 72 h after infection. Coomassie blue staining of an SDS-PAGE analysis, showing relative quantities of protein samples loaded. Lanes 1 and 4 contain the samples of the Ad5/RSV/GL2-infected cells. Lanes 2 and 3 contain the samples of the cells infected with two individual stocks of Ad5CMV-p53 at 24 h after infection. Lanes 5 and 6 are the Ad5CMV-p53-infected cell samples collected at 72 h after infection. Lane 7 is mock-infected H358 sample 72 h after infection. Lane M, prestained molecular weight markers in kDa (GIBCO-BRL).

FIG. 5B. Western blot analysis of the identical lane setting gel as that f the SDS-PAGE in FIG. 5A. The relative levels

of expression of p53 were analyzed by Western blotting using anti-p53. Primary antibodies used were monoclonal antibodies against p53 protein (PAb 1801, Oncogene Science Inc.) and β-actin (Amersham Inc.). The HRP-conjugated second antibody and ECL developer were from Amersham Inc.viral-infected H358 cells by Western Blotting. Western blot of FIG. 5B have an equivalent setup and order to those in FIG. 5A.

FIGS. 6A–B. Time course of the p53 expression, determined by Western blotting (FIG. 6B). Multiple dishes of H358 cells were infected with Ad5CMV-p53 at 10 PFU/cell. Cell lysates were prepared at indicated time points after infection. Western blotting was probed with anti-p53 and anti-actin antibodies simultaneously. The lanes designated 'C' represent negative controls. The histogram represents the relative quantities of p53 as determined by a densitometer (FIG. 6A).

FIG. 7A. Growth curve of virally-infected human lung cancer cells of cell lines H358. Cells were inoculated at 10^5 cells per dish (60 mm) and 6 dishes per cell line. After 24 hours, the cells were infected with Ad5CMV-p53 or Ad5/RSV/GL2 at 10 m.o.i. (Multiplicity of infection, i.e., PFU/cell). After infection cells were counted daily for 6 days. The growth curves represent data obtained from 4 separate studies.

FIG. 7B. Growth curve of virally-infected human lung cancer cells of cell line H322. Cells were inoculated at 10^5 cells per dish (60 mm) and 6 dishes per cell line. After 24 hours, the cells were infected with Ad5CMV-p53 or Ad5/RSV/GL2 at 10 m.o.i. (Multiplicity of infection, i.e., PFU/cell). After infection cells were counted daily for 6 days. The growth curves represent data obtained from 4 separate studies.

FIG. 7C. Growth curve of virally-infected human lung cancer cells of cell line H460. Cells were inoculated at 10^5 cells per dish (60 mm) and 6 dishes per cell line. After 24 hours, the cells were infected with Ad5CMV-p53 or Ad5/RSV/GL2 at 10 m.o.i. (Multiplicity of infection, i.e., PFU/cell). After infection cells were counted daily for 6 days. The growth curves represent data obtained from 4 separate studies.

FIG. 8. Flow chart of tests of Ad5CMV-p53 in orthotopic lung cancer model. The dosages and schedule of treatment of nude mice inoculated with H226Br cells and viruses are summarized in the flow chart.

FIGS. 9A, 9B, 9C, and 9D. Samples of the lung and mediastinum dissection from treated and control mice. FIGS. 9A, 9B, 9C, and 9D are four panels of a single figure. The mice were sacrificed at the end of the 6-week posttreatment period. The lung and mediastinum tissues were dissected for evaluation of tumor formation. FIG. 9A is a sample of mediastinal block from a normal nude mice; FIG. 9B is the mediastinal block sample from the vehicle (PBS)-treated mice; FIG. 9C is the mediastinal block sample from the Ad5CMV-p53-treated mice; FIG. 9D is the mediastinal block sample from the Ad5/RSV/GL2-treated mice. Arrows indicate the tumor masses.

FIG. 10A. The effects of continuous exposure to CDDP on the growth rates of parental, Ad-Luc-infected, and Ad-p53-infected H358 cells. H358 cells (1.5×10^5 cells/well) were seeded in duplicate n a 24-well plate. After 24 hours, 100 µl of medium, Ad-Luc viral stock (10^8 PFU/ml), or Ad-p53 viral stock (10^8 PFU/ml) was added. Following an additional 24-hour incubation, the medium that contained virus was replaced with fresh medium that contained 10 µg/ml of CDDP.

FIG. 10B. 24-hour exposure to CDDP on the growth rates of parental, Ad-Luc-infected, and Ad-p53-infected H358 cells. Cells were exposed to CDDP (FIG. 10A) continuously or (FIG. 10B) for 24 hours followed by recovery in drug-free medium. Cells that remained as an attached monolayer were assessed for viability over 5 days by measuring trypan blue uptake. The mean +/– SE is shown. The day 5 cell number for the Ad-p53:CDDP group differs significantly from all other groups for both A and B ($p<0.05$ by Student's t-test).

FIG. 10C. The effects of different concentrations of CDDP on the viability of Ad-p53-infected H358 cells. After 24-hour exposure to the Ad-Luc or Ad-p53 virus, cells were treated with 0, 10, or 100 µg/ml of CDDP for 24 hours and then assessed for viability.

FIG. 11A. Nucleosomal DNA fragmentation in Ad-p53-infected H358 cells exposed to CDDP. Cells were infected and treated with CDDP for 24 hours as described in the legend to FIG. 10.

FIGS. 11B, 11C, 11D, 11E, 11F and 11G. H358 cells that were grown on chamber slides, infected with Ad-p53 for 24 hours, treated with CDDP for an additional 24 hours, and fixed for in situ labeling of DNA fragmentation. Pictured are parental H358 cells (B) without or (C) with CDDP; Ad-Luc-infected cells (D) without or (E) with CDDP; and Ad-p53-infected cells (F) without or (G) with CDDP. The arrowhead shows an example of darkly stained nuclear fragments. Bar=100 µm.

FIG. 12A. Effect of the combination of Ad-p53 infection with CDDP treatment on H358 tumor spheroids. Multicellular tumor spheroids of H358 cells were prepared as previously described (Takahashi, et al. (1989)). On day 0, spheroids with a diameter of 150 to 200 µm were placed in a 24-well agar coated plate and exposed to Ad-p53 or Ad-Luc for 24 hours. On day 1, medium with 10 µg/ml of CDDP was added following removal of virus-containing medium. On day 2, after a 24-hour incubation, the overlay was replaced with 1 ml of fresh drug-free medium. The perpendicular diameters were measured using an inverted microscope. The relative volume change was calculated according to the formula $a^2\times b / a_1^2\times b_1$, where a and b are the smallest and largest diameters of the spheroid, respectively, and a_1 and b_1 are the diameters on day 1. Only the relative volume of the Ad-p53/CDDP spheroids is significantly less ($p<0.05$ by Student's t-test) than the control group (Ctl).

FIGS. 12B, 12C, 12D, and 12E. In situ dUTP labeling with TdT for detection of apoptosis. H358 spheroids were fixed on day 3 and stained as described in Materials and Methods of Example 7. (B) Control untreated spheroid. (C) spheroid treated with CDDP. (D) Ad-p53-infected spheroid, and (E) Ad-p53-infected spheroid treated with CDDP. Bar=100 µm.

FIGS. 13A-1, 13A-2. Induction of apoptosis by CDDP after in vivo infection with Ad-p53 as measured by tumor volume changes. H358 cells (5×10^6) in 0.1 ml Hank's balanced salt solution were injected subcutaneously into the right flank of BALB/c female nu/nu mice. Thirty days later, 200 µl of medium alone or medium containing Ad-Luc (10^8 PFU/ml) or Ad-p53 (10^8 PFU/ml) was injected into tumors with a diameter of 5 to 6 mm. Intratumoral injection (100 µl) and peritumoral injection in two opposite sites (50 µl each) were performed. CDDP (3 mg/kg) or control physiological saline was given intraperitoneally. The tumors were measured with calipers in two perpendicular diameters without the knowledge of the treatment groups, and a tumor volume was calculated by assuming a spherical shape with the average tumor diameter calculated as the square root of the

product of cross-sectional diameters. Five mice were used for each treatment group and the mean +/− SE is shown. The data was analyzed using the Student's t-test. The arrow shows the day of treatment. Two independent determinations are shown. $p<0.05$ from day 5 in test 1; $p<0.05$ from day 7 in test 2. (B–E)

FIGS. 13B, 13C, 13D, and 13E. Histologic study using the TdT-mediated biotin-dUTP labeling technique. Tumors were harvested 5 days after the beginning of treatment and immediately embedded into O. C. T. compound. Frozen tissues were cut in a cryostat at 5-μm thicknesses. The sections were treated with 1 μg/ml proteinase K and stained as described in the legend to FIG. 12. Pictured are d to FIG. 12. Pictured are H358 tumors treated with (B) CDDP alone. (C) Ad-p53 alone. or (D. E) Ad-p53 in the combination with CDDP. Bars=0.5 mm. All animal care was in accordance with the UT M. D. Anderson Institutional Animal Care and Use Committee.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

A. Molecular Events in Lung Cancer Development

Studies carried out by the present inventors has identified critical molecular events leading to the development and progression of cancer. This enabled the inventors to develop new methods for restoring certain normal protein functions so that the malignant phenotype can be suppressed in vivo.

The most common lung cancer histologies (80%) are grouped under the term non-small-cell lung cancer (NSCLC) and include squamous, adenocarcinoma, and large-cell undifferentiated. Many of the current data on the molecular biology of lung cancer come from the study of the more uncommon small-cell lung cancer (SCLC). SCLC can be distinguished from NSCLC by the neuroendocrine features of the cells; SCLC is very responsive to chemotherapy but recurs rapidly after treatment. NSCLC also may serve as a model for other carcinogen-induced epithelial cancers. The approaches and observations developed in this study may be applicable to other types of epithelial cancers.

Abundant evidence has accumulated that the process of malignant transformation is mediated by a genetic paradigm. The major lesions detected in cancer cells occur in dominant oncogenes and tumor suppressor genes. Dominant oncogenes have alterations in a class of genes called proto-oncogenes, which participate in critical normal cell functions, including signal transduction and transcription. Primary modifications in the dominant oncogenes that confer the ability to transform include point mutations, translocations, rearrangements, and amplification. Tumor suppressor genes appear to require homozygous loss of function, by mutation, deletion, or a combination of these for transformation to occur. Some tumor suppressor genes appear to play a role in the governance of proliferation by regulation of transcription. Modification of the expression of dominant and tumor suppressor oncogenes is likely to influence certain characteristics of cells that contribute to the malignant phenotype.

Despite increasing knowledge of the mechanisms involved in oncogene-mediated transformation, little progress has occurred in developing therapeutic strategies that specifically target oncogenes and their products. Initially, research in this area was focused on dominant oncogenes, as these were the first to be characterized. DNA-mediated gene transfer studies showed acquisition of the malignant phenotype by normal cells following the transfer of DNA from malignant human tumors.

B. p53 and p53 Mutations in Cancer

P53 is currently recognized as a tumor suppressor gene (Montenarh, 1992). High levels have been found in many cells transformed by chemical carcinogenesis, ultraviolet radiation, and several viruses, including SV40. The p53 gene is a frequent target of mutational inactivation in a wide variety of human tumors and is already documented to be the most frequently-mutated gene in common human cancers (Mercer, 1992). It is mutated in over 50% of human NSCLC (Hollestein et al., 1991) and in a wide spectrum of other tumors.

The p53 gene encodes a 375-amino-acid phosphoprotein that can form complexes with host proteins such as large-T antigen and E1B. The protein is found in normal tissues and cells, but at concentrations which are minute by comparison with transformed cells or tumor tissue. Interestingly, wild-type p53 appears to be important in regulating cell growth and division. Overexpression of wild-type p53 has been shown in some cases to be anti-proliferative in human tumor cell lines. Thus p53 can act as a negative regulator of cell growth (Weinberg, 1991) and may directly suppress uncontrolled cell growth or indirectly activate genes that suppress this growth. Thus, absence or inactivation of wild type p53 may contribute to transformation. However, some studies indicate that the presence of mutant p53 may be necessary for full expression of the transforming potential of the gene.

Although wild-type p53 is recognized as a centrally important growth regulator in many cell types, its genetic and biochemical traits appear to have a role as well. Missense mutations are common for the p53 gene and are essential for the transforming ability of the oncogene. A single genetic change prompted by point mutations can create carcinogenic p53. Unlike other oncogenes, however, p53 point mutations are known to occur in at least 30 distinct codons, often creating dominant alleles that produce shifts in cell phenotype without a reduction to homozygosity. Additionally, many of these dominant negative alleles appear to be tolerated in the organism and passed on in the germ line. Various mutant alleles appear to range from minimally dysfunctional to strongly penetrant, dominant negative alleles (Weinberg, 1991).

Casey and colleagues have reported that transfection of DNA encoding wild-type p53 into two human breast cancer cell lines restores growth suppression control in such cells (Casey et al., 1991). A similar effect has also been demonstrated on transfection of wild-type, but not mutant, p53 into human lung cancer cell lines (Takahasi et al., 1992). The p53 appears dominant over the mutant gene and will select against proliferation when transfected into cells with the mutant gene. Normal expression of the transfected p53 does not affect the growth of cells with endogenous p53. Thus, such constructs might be taken up by normal cells without adverse effects.

It is thus possible that the treatment of p53-associated cancers with wild type p53 may reduce the number of malignant cells. However, studies such as those described above are far from achieving such a goal, not least because DNA transfection cannot be employed to introduce DNA into cancer cells within a patients' body.

C. Gene Therapy Techniques

There have been several experimental approaches to gene therapy proposed to date, but each suffer from their particular drawbacks (Mulligan, 1993). As mentioned above, basic transfection methods exist in which DNA containing the gene of interest is introduced into cells non-biologically, for

example, by permeabilizing the cell membrane physically or chemically. Naturally, this approach is limited to cells that can be temporarily removed from the body and can tolerate the cytotoxicity of the treatment, i.e. lymphocytes. Liposomes or protein conjugates formed with certain lipids and amphophilic peptides can be used for transfection, but the efficiency of gene integration is still very low, on the order of one integration event per 1,000 to 100,000 cells, and expression of transfected genes is often limited to days in proliferating cells or weeks in non proliferating cells. DNA transfection is clearly, therefore, not a suitable method for cancer treatment.

A second approach capitalizes on the natural ability of viruses to enter cells, bringing their own genetic material with them. Retroviruses have promise as gene delivery vectors due to their ability to integrate their genes into the host genome, transferring a large amount of foreign genetic material, infecting a broad spectrum of species and cell types and of being packaged in special cell-lines. However, three major problems hamper the practical use of retrovirus vectors. First, retroviral infectivity depends on the availability of the viral receptors on the target surface. Second, retroviruses only integrate efficiently into replicating cells. And finally, retroviruses are difficult to concentrate and purify.

D. Adenovirus Constructs for use in Gene Therapy

Human adenoviruses are double-stranded DNA tumor viruses with genome sizes of approximate 36 kb (Tooza, 1981). As a model system for eukaryotic gene expression, adenoviruses have been widely studied and well characterized, which makes them an attractive system for development of adenovirus as a gene transfer system. This group of viruses is easy to grow and manipulate, and they exhibit a broad host range in vitro and in vivo. In lytically infected cells, adenoviruses are capable of shutting off host protein synthesis, directing cellular machineries to synthesize large quantities of viral proteins, and producing copious amounts of virus.

The E1 region of the genome includes E1A and E1E which encode proteins responsible for transcription regulation of the viral genome, as well as a few cellular genes. E2 expression, including E2A and E2B, allows synthesis of viral replicative functions, e.g. DNA-binding protein, DNA polymerase, and a terminal protein that primes replication. E3 gene products prevent cytolysis by cytotoxic T cells and tumor necrosis factor and appear to be important for viral propagation. Functions associated with the E4 proteins include DNA replication, late gene expression, and host cell shutoff. The late gene products include most of the virion capsid proteins, and these are expressed only after most of the processing of a single primary transcript from the major late promoter has occurred. The major late promoter (MLP) exhibits high efficiency during the late phase of the infection (Stratford-Perricaudet and Perricaudet, 1991a).

As only a small portion of the viral genome appears to be required in cis (Tooza, 1981), adenovirus-derived vectors offer excellent potential for the substitution of large DNA fragments when used in connection with cell lines such as 293 cells. Ad5-transformed human embryonic kidney cell line (Graham, et al., 1977) have been developed to provide the essential viral proteins in trans. The inventors thus reasoned that the characteristics of adenoviruses rendered them good candidates for use in targeting cancer cells in vivo (Grunhaus & Horwitz, 1992).

Particular advantages of an adenovirus system for delivering foreign proteins to a cell include (i) the ability to substitute relatively large pieces of viral DNA by foreign DNA; (ii) the structural stability of recombinant adenoviruses; (iii) the safety of adenoviral administration to humans; and (iv) lack of any known association of adenoviral infection with cancer or malignancies; (v) the ability to obtain high titers of the recombinant virus; and (vi) the high infectivity of Adenovirus.

Further advantages of adenovirus vectors over retroviruses include the higher levels of gene expression. Additionally, adenovirus replication is independent of host gene replication, unlike retroviral sequences. Because adenovirus transforming genes in the E1 region can be readily deleted and still provide efficient expression vectors, oncogenic risk from adenovirus vectors is thought to be negligible (Grunhaus & Horwitz, 1992).

In general, adenovirus gene transfer systems are based upon recombinant, engineered adenovirus which is rendered replication-incompetent by deletion of a portion of its genome, such as E1, and yet still retains its competency for infection. Relatively large foreign proteins can be expressed when additional deletions are made in the adenovirus genome. For example, adenoviruses deleted in both E1 and E3 regions are capable of carrying up to 10 Kb of foreign DNA and can be grown to high titers in 293 cells (Stratford-Perricaudet and Perricaudet, 1991a). Surprisingly persistent expression of transgenes following adenoviral infection has also been reported.

Adenovirus-mediated gene transfer has recently been investigated as a means of mediating gene transfer into eukaryotic cells and into whole animals. For example, in treating mice with the rare recessive genetic disorder ornithine transcarbamylase (OTC) deficiency, it was found that adenoviral constructs could be employed to supply the normal OTC enzyme. Unfortunately, the expression of normal levels of OTC was only achieved in 4 out of 17 instances (Stratford-Perricaudet et al., 1991b). Therefore, the defect was only partially corrected in most of the mice and led to no physiological or phenotypic change. These type of results therefore offer little encouragement for the use of adenoviral vectors in cancer therapy.

Attempts to use adenovirus to transfer the gene for cystic fibrosis transmembrane conductance regulator (CFTR) into the pulmonary epithelium of cotton rats have also been partially successful, although it has not been possible to assess the biological activity of the transferred gene in the epithelium of the animals (Rosenfeld et al., 1992). Again, these studies demonstrated gene transfer and expression of the CFTR protein in lung airway cells but showed no physiologic effect. In the 1991 Science article, Rosenfeld et al. showed lung expression of α1-antitrypsin protein but again showed no physiologic effect. In fact, they estimated that the levels of expression that they observed were only about 2% of the level required for protection of the lung in humans, i.e., far below that necessary for a physiologic effect.

The gene for human α_1-antitrypsin has been introduced into the liver of normal rats by intraportal injection, where it was expressed and resulted in the secretion of the introduced human protein into the plasma of these rats (Jaffe et al., 1992). However, and disappointingly, the levels that were obtained were not high enough to be of therapeutic value.

These type of results do not demonstrate that adenovirus is able to direct the expression of sufficient protein in recombinant cells to achieve a physiologically relevant effect, and they do not, therefore, suggest a usefulness of the

adenovirus system for use in connection with cancer therapy. Furthermore, prior to the present invention, it was thought that p53 could not be incorporated into a packaging cell, such as those used to prepare adenovirus, as it would be toxic. As E1B of adenovirus binds to p53, this was thought to be a further reason why adenovirus and p53 technology could not be combined.

E. p53-Adenovirus Constructs and Tumor Suppression

The present invention provides cancer gene therapy with a new and more effective tumor suppressor vector. This recombinant virus exploits the advantages of adenoviral vectors, such as high titer, broad target range, efficient transduction, and non-integration in target cells. In one embodiment of the invention, a replication-defective, helper-independent adenovirus is created that expresses wild type p53 (Ad5CMV-p53) under the control of the human cytomegalovirus promoter.

Control functions on expression vectors are often provided from viruses when expression is desired in mammalian cells. For example, commonly used promoters are derived from polyoma, adenovirus 2 and simian virus 40 (SV40). The early and late promoters of SV40 virus are particularly useful because both are obtained easily from the virus as a fragment which also contains the SV40 viral origin of replication. Smaller or larger SV40 fragments may also be used provided there is included the approximately 250 bp sequence extending from the HindIII site toward the BglI site located in the viral origin of replication. Further, it is also possible, and often desirable, to utilize promoter or control sequences normally associated with the included gene sequence, provided such control sequences are compatible with the host cell systems.

An origin of replication may be provided by construction of the vector to include an exogenous origin, such as may be derived from SV40 or other viral (e.g., polyoma, adeno, VSV, BPV) source, or may be provided by the host cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the latter is often sufficient.

The design and propagation of the preferred p53 adenovirus is diagramed in FIG. 1. In connection with this, an improved protocol has been developed for propagating and identifying recombinant adenovirus (discussed below). After identification, the p53 recombinant adenovirus was structurally confirmed by the PCR analysis, as indicated in FIG. 2. After isolation and confirmation of its structure, the p53 adenovirus was used to infect human lung cancer cell line H358, which has a homozygous p53 gene deletion. Western blots showed that the exogenous p53 protein was expressed at a high level (FIG. 4 and FIG. 5) and peaked at day 3 after infection (FIG. 6).

It was also shown in a p53 point mutation cell line H322 that the mutant p53 was down regulated by the expression of the exogenous p53. As an experimental control, a virion (Ad5/RSV/GL2) that had a structural similarity to that of Ad5CMV-p53 was used. This virion contained a luciferase CDNA driven by Rous sarcoma virus LTR promoter in the expression cassette of the virion. Neither p53 expression nor change in actin expression was detected in cells infected by the virion Ad5/RSV/GL2. Growth of the H358 cells infected with Ad5CMV-p53 was greatly inhibited in contrast to that of noninfected cells or the cells infected with the control virion (FIG. 7A). Growth of H322 cells was also greatly inhibited by the p53 virion (FIG. 7B), while that of human lung cancer H460 cells containing wild-type p53 was less affected (FIG. 7C).

Ad5CMV-p53 mediated a strong inhibitory effect on lung cancer cell growth in vitro. Growth inhibition was not as evident when the cells were infected with Ad5CMV-p53 at MOI lower than 1 PFU/cell, whereas, at MOI higher than 100 PFU/cell, cytotoxicity could be observed even with control virus Ad5/RSV/GL2. In our studies, the optimal dose for growth rate studies was 10–50 PFU/cell. Within this dose range, cell growth inhibition was attributable to the expressed p53 protein.

Tests in nude mice demonstrated that tumorigenicity of the Ad5CMV-p53-treated H358 cells was greatly inhibited. In a mouse model of orthotopic human lung cancer, the tumorigenic H226Br cells, with a point mutation in p53, were inoculated intratracheally 3 days prior to the virus treatment. Intratracheal instillation of Ad5CMV-p53 prevented tumor formation in this model system suggesting that the modified adenovirus is an efficient vector for mediating transfer and expression of tumor suppressor genes in human cancer cells and that the Ad5CMV-p53 virus may be further developed into a therapeutic agent for use in cancer gene therapy.

Ad5CMV-p53 mediated a high level of expression of the p53 gene in human lung cancer cells as demonstrated by Western blot analysis. Exogenous p53 protein was approximately 14 times more abundant than the endogenous wild-type p53 in H460 cells and about two to four times more abundant than the β-actin internal control in H358 cells. The high level of expression may be attributed to (1) highly efficient gene transfer, (2) strong CMV promoter driving the p53 CDNA, and (3) adenoviral E1 enhancer enhancing the p53 CDNA transcription. The duration of p53 expression after infection was more than 15 days in H358 cells. However, there was a rapid decrease in expression after postinfection day 5. PCR analysis of the DNA samples from the infected H358 cells showed a decrease of the viral DNA level with the decreased protein level, indicating the loss of viral DNA during the continuous growth of cancer cells in vitro.

The decrease in p53 expression may also have resulted from cellular attenuation of the CMV promoter that controls p53 expression, since the phenomenon of host cell-mediated CMV promoter shut off has been reported previously (Dai, et al., 1992). Adenoviral vectors are nonintegrative gene transfer vectors and therefore the duration of gene expression depends upon a number of factors, including the host cells, the genes transferred, and the relevant promoter. Crystal and co-workers showed low level expression of the cystic fibrosis transmembrane conductance regulator gene in cotton rat epithelial cells was detectable 6 weeks after infection (Rosenfeld, et al., 1992). Perricaudet's laboratory demonstrated minimal expression of minidystrophin gene in mdx mouse muscle lasted for more than 3 months after infection. The short-term high level expression of the wild-type p53 protein observed in the present study may have the beneficial effect of reducing possible side effects on normal cells following in vivo treatment with Ad5CMV-p53.

The studies disclosed herein indicate that the p53 recombinant adenovirus possesses properties of tumor suppression, which appear to operate by restoring p53 protein function in tumor cells. These results provide support for the use of the Ad5CMV-p53 virion as a therapeutic agent for cancer treatment.

F. DNA Damaging Agents

A wide variety f DNA damaging agents may be used with the present invention, such as, agents that directly

crosslink DNA, agents that intercalate into DNA, and agents that lead to chromosomal and mitotic aberrations by affecting nucleic acid synthesis.

Agents that directly cross-link nucleic acids, specifically DNA, are envisaged and are shown herein, to eventuate DNA damage leading to a synergistic antineoplastic combination. Agents such as cisplatin, and other DNA alkylating may be used. Cisplatin has been widely used to treat cancer, with efficacious doses used in clinical applications of 20 mg/m^2 for 5 days every three weeks for a total of three courses. Cisplatin is not absorbed orally and must therefore be delivered via injection intravenously, subcutaneously, intratumorally or intraperitoneally.

Agents that damage DNA also include compounds that interfere with DNA replication, mitosis, and chromosomal segregation. Examples of these compounds include adriamycin, also known as doxorubicin, etoposide, verapamil, podophyllotoxin, and the like. Widely used in clinical setting for the treatment of neoplasms these compounds are administered through bolus injections intravenously at doses ranging from 25–75 mg/m^2 at 21 day intervals for adriamycin, to 35–50 mg/m2 for etoposide intravenously or double the intravenous dose orally.

Agents that disrupt the synthesis and fidelity of nucleic acid precursors, and subunits also lead to DNA damage. As such a number of nucleic acid precursors have been developed. Particularly useful are agents that have undergone extensive testing and are readily available. As such, agents such as 5-fluorouracil (5-FU), are preferentially used by neoplastic tissue, making this agent particularly useful for targeting to neoplastic cells. Although quite toxic, 5-FU, is applicable in a wide range of carriers, including topical, however intravenous administration with doses ranging from 3 to 15 mg/kg/day being commonly used.

Other factors that cause DNA damage and have been used extensively include what are commonly known as γ-rays, X-rays, and/or the directed delivery of radioisotopes to tumor cells. Other forms of DNA damaging factors are also contemplated such as microwaves and UV-irradiation. It is most likely that these all of these factors effect a broad range of damage on the precursors of DNA, the replication and repair of DNA, and the assembly and maintenance of chromosomes. Dosage ranges for X-rays range from daily doses of 50 to 200 roentgens for prolonged periods of time (3 to 4 weeks), to single doses of 2000 to 6000 roentgens. Dosage ranges for radioisotopes vary widely, and depend on the half-life of the isotope, the strength and type of radiation emitted, and the uptake by the neoplastic cells.

The skilled artisan in directed to "Remington's Pharmaceutical Sciences" 15th Edition, chapter 33, in particular pages 624–652. Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

G. p53 and Cisplatin Treatment

In an effort to determine the efficacy of a combination of gene replacement therapy and chemotherapy in human cancer, the inventors examined whether sequential administration of Ad-p53 and CDDP could induce apoptosis in vivo. Following 3 days of direct intratumoral injection of Ad-p53 or intraperitoneal administration of CDDP, H358 tumors implanted subcutaneously in nu/nu mice showed a modest slowing of growth. However, if Ad-p53 and CDDP were simultaneously administered, tumors partially regressed and the tumor size remained statistically significantly smaller than those in any of the other treatment groups. The growth inhibitory effect was even more pronounced after two treatment cycles (FIG. 13A). Histologic examination revealed a massive destruction of tumor cells in the area where Ad-p53 was injected in mice treated with CDDP. In situ staining demonstrated many apoptotic cells around acellular spaces (FIGS. 13B–E). In contrast, tumors treated with CDDP alone or Ad-p53 alone showed neither acellularity nor apoptotic areas.

The present invention describes a novel strategy for human gene therapy combined with conventional chemotherapy using a DNA crosslinking agent. Tumor cell resistance to chemotherapeutic drugs represents a major problem in clinical oncology. NSCLC accounts for at least 80% of the cases of lung cancer; patients with NSCLC are, however, generally unresponsive to chemotherapy (Doyle, 1993). One goal of current cancer research is to find ways to improve the efficacy of gene replacement therapy for cancer by investigating interaction between the gene product and chemotherapeutic drugs. The herpes simplex-thymidine kinase (HS-tK) gene, when delivered to brain tumors by a retroviral vector system, successfully induced susceptibility to the antiviral agent ganciclovir (Culver, et al., 1992). The HS-tK gene product is an exogenous viral enzyme, whereas the wt-p53 protein is expressed in normal tissues, suggesting that the modulation of chemoresistance by alterations in wt-p53 expression might be an alternative approach using a pathway mediated by an endogenous genetic program.

An adenovirus system has potential advantages for gene delivery in vivo, such as ease of producing high titer virus, high infection efficiency, and infectivity for many types of cells. The stability and duration of expression of the introduced gene are still controversial, however. For chemo-gene therapy, the levels of expression and the high infectivity may be are more significant than the duration of expression, because drugs can kill infected cells within several days. The increase in p53 levels in cells that are sensitive to chemotherapeutic drugs can occur within 6 hours after DNA-damaging stimuli (Fritsche, et al., 1993, Zhan, et al., 1993), although increased p53 DNA binding activity can be reversed over the course of 4 hours if the stimulus is removed (Tishler, et al., 1993). In the present model, the expression of the wt-p53 gene is driven independently by the cytomegalovirus promoter contained in an Ad-p53 vector. Therefore, a high level of p53 expression can be maintained even after cessation of drug exposure. The expression of wt-p53 protein by Ad-p53 peaks at postinfection day 3 (14-fold greater than endogenous wild type) and decreases to a low level by day 9 (Zhang, et al., 1993). This suggests that a transiently high level of wt-p53 expression is sufficient to initiate the cytotoxic program in the cancer cell.

H. Patients and Treatment Protocols

The inventors propose that the regional delivery of adenoviral-p53 gene constructs to lung cancer cells in patients with p53-linked cancers, such as unresectable obstructing endobronchial cancers, will be a very efficient method for delivering a therapeutically effective gene to counteract the clinical disease. The deliver of the p53 gene is to occur in combination with agents or factors that lead to DNA damage. This combined approach is a significant improvement on current cancer therapies, for example the loss of sensitivity to cisplatin alone, which rely on attempts to kill or remove the last cancer cell by effecting DNA

damage. As tumor cell dormancy is an established phenomenon, this makes effective killing highly unlikely.

It is anticipated that the uptake of the adenovirus constructs by NSCLC cells will decrease the rate of proliferation of these cells, however, the present examples demonstrate that the combined use of a DNA damaging agent or factor with the p53 adenovirus leads to a profound diminution of cell growth and tumor size, not shown with either factor alone. The compositions and methods disclosed herein, strongly portend an increase in the length of time the affected lung would remain expanded, prevent regrowth of the tumor and division of tumor cells, and prolong the patient's survival.

Patients with unresectable endobronchial tumor recurrence that is partially or completely obstructing the airway and that have failed or are unable to receive external beam radiotherapy will be considered for this combined protocol. Existing therapies for this condition offer only short-term palliation. Most patients have recurred despite external beam radiotherapy. It may be possible to insert a brachytherapy catheter and administer additional radiotherapy, intravenous administration of DNA damaging agents. Patients receiving current treatments have a median survival of 6 months. Patients failing brachytherapy would also be eligible to receive gene therapy. Tumor can be removed from the airway with the laser or biopsy forceps. This can be done in conjunction with injection of the adenoviral constructs thus decreasing the volume that must be injected. The administration of the viral constructs would not preclude the patient from receiving other palliative therapy if the tumor progresses.

I. Other Gene Transfer Techniques

Successful gene therapy generally requires the integration of a gene able to correct the genetic disorder into the host genome, where it would co-exist and replicate with the host DNA and be expressed at a level to compensate for the defective gene. Ideally, the disease would be cured by one or a few treatments, with no serious side effects. There have been several approaches to gene therapy proposed to date, which may be used with the present invention.

A first approach is to transfect DNA containing the gene of interest into cells, e.g., by permeabilizing the cell membrane either chemically or physically. This approach is generally limited to cells that can be temporarily removed from the body and can tolerate the cytotoxicity of the treatment (i.e. lymphocytes). Liposomes or protein conjugates formed with certain lipids and amphophilic peptides can be used for in vivo transfection (Stewart et al., 1992; Torchilin et al., 1992; Zhu et al., 1993), however present efficiency of gene integration is very low. It is estimated that the gene of interest integrates into the genome of only one cell in 1,000 to 100,000. In the absence of integration, expression of the transfected gene is limited to several days in proliferating cells or several weeks in non proliferating cells due to the degradation of the unintegrated DNAs.

A second approach capitalizes on the natural ability of viruses to enter cells, bringing their own genetic material with them. Retroviruses have promise as gene delivery vectors due to their ability to integrate their genes into the host genome, transferring a large amount of foreign genetic material, infecting a broad spectrum f species and cell types and of being packaged in special cell-lines (Miller, 1992).

A third method uses other viruses, such as adenovirus, herpes simplex viruses (HSV), cytomegalovirus (CMV), and adenoassociated virus (AAV), which are engineered to serve as vectors for gene transfer. Although some viruses that can accept foreign genetic material are limited in the number of nucleotides they can accommodate and in the range of cells they infect, these viruses have been demonstrated to successfully effect gene expression. However, adenoviruses do not integrate their genetic material into the host genome and therefore do not require host replication for gene expression, making them ideally suited for rapid, efficient, heterologous gene expression.

Even though the invention has been described with a certain degree of particularity, it is evident that many alternatives, modifications, and variations will be apparent to those skilled in the art in light of the foregoing disclosure. Accordingly, it is intended that all such alternatives, modifications, and variations which fall within the spirit and the scope of the invention be embraced by the defined claims.

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1

Construction of p53 Expression Vector

This example describes the construction of a p53 expression vector. This vector is constructed as indicated and is used to replace the E1 region (1.3–9.2 m.u.) of the Adenovirus strain Ad5 genome and employed to construct the Adenovirus virion described in Example 2.

The p53 expression cassette shown in FIG. 1, which contains human cytomegalovirus (CMV) promoter (Boshart, et al., 1985), p53 cDNA, and SV40 early polyadenylation signal, was inserted between the Xba I and Cla I sites of pXCJL1 (provided by Dr. Frank L. Graham, McMaster University, Canada).

The genome size is about 35.4 kb, divided into 100 map units (1 m.u.=0.35 kb). The p53 expression cassette replaced the E1 region (1.3–9.2 m.u.) of the Ad5 genome.

Primer 1 has the sequence 5'-GGCCCACCCCCTTGGCTTC-3' (SEQ ID NO:1) and is located in the first intron downstream of the human CMV major IE gene promoter (Boshart, et al., 1985). Primer 2 has the sequence 5'-TTGTAACCATTATAAGCTGC-3' (SEQ ID NO:2) and is located in SV40 early polyadenylation signal. Both of the primers, 15–20 bp away from the p53 cDNA insert at both ends, define a 1.40 kb PCR product. Primer 3 has the sequence 5'-TCGTTTCTCAGCAGCTGTTG-3' (SEQ ID NO:3) and primer 4 has the sequence 5'-CATCTGAACTCAAAGCGTGG-3' (SEQ ID NO:4) and are located at 11 m.u. and 13.4 m.u. of the Ad5 genome, respectively, which define a 0.86 kb viral-genome specific PCR product.

EXAMPLE 2

Generation and Propagation of Recombinant p53 Adenovirus

This example describes one method suitable for generating helper-independent recombinant adenoviruses express-

ing p53. The molecular strategy employed to produce recombinant adenovirus is based upon the fact that, due to the packaging limit of adenovirus, pJM17 cannot form virus on its own. Therefore, homologous recombination between the p53 expression vector plasmid and pJM17 within a transfected cell results in a viable virus that can be packaged only in cells which express the necessary adenoviral proteins.

The method of this example utilizes 293 cells as host cells to propagate viruses that contain substitutions of heterologous DNA expression cassettes at the E1 or E3 regions. This process requires cotransfection of DNA into 293 cells. The transfection largely determines efficiency of viral propagation. The method used for transfection of DNA into 293 cells prior to the present invention was usually calcium-phosphate/DNA coprecipitation (Graham and van der Eb, 1973). However, this method together with the plaque assay is relatively difficult and typically results in low efficiency of viral propagation. As illustrated in this example, transfection and subsequent identification of infected cells were significantly improved by using liposomemediated transfection, when identifying the transfected cells by cytopathic effect (CPE).

The 293 cell line was maintained in Dulbecco's modified minimal essential medium supplemented with 10% heat-inactivated horse serum. The p53 expression vector and the plasmid pJM17 (McGrory, et al., 1988) for homologous recombination were cotransfected into 293 cells by DOTAP-mediated transfection according to the manufacture's protocol (Boehringer Mannheim Biochemicals, 1992). This is schematically shown in FIG. 1.

The 293 cells (passage 35, 60% confluency) were inoculated 24 hours prior to the transfection in either 60 mm dishes or 24-well plates. The cells in each well were transfected with: 30 μl DOTAP, 2 μg of p53 expression vector, and 3 μg of plasmid pJM17. After transfection cells were fed with the MEM medium every 2–3 days until the onset of CPE.

EXAMPLE 3

Confirming the Identity of Recombinant Adenovirus

This example illustrates a new polymerase chain reaction (PCR) assay for confirming the identity of recombinant virions following cotransfection of the appropriate cell line.

Aliquots of cell culture supernatants (50 to 370 μl) were collected from the test plates, treated with proteinase K (50 μg/ml with 0.5% SDS and 20 mM EDTA) at 56° C. for 1 hour, extracted with phenol-chloroform, and the nucleic acids were ethanol precipitated. The DNA pellets were resuspended in 20 μl dH_2O and used as template for PCR amplification. The relative locations of the PCR primers and their sequences are depicted in FIG. 1 and are SEQ ID NOS:1, 2, 3 and 4, respectively. The cDNA insert-specific primers define a 1.4 kb PCR product and the viral genome-specific primers define a 0.86 kb PCR product. The PCR reactions were carried out in a 50 μl volume containing 4 mM $MgCl_2$, 50 mM KCl, 0.1% triton X-100, 200 μM each of dNTPs, 10 mM Tris-Cl (pH 9.0), 2 μM of each primer, and 1.0 unit of Taq polymerase (Promega). The reactions were carried out at 94° C., 0.5 min, 56° C., 0.5 min, and 72° C., 1 min for 30 cycles.

In order t simplify the procedure of identification of newly propagated recombinant virus, a direct PCR assay n DNA samples from cell culture supernatant was developed. Aliquots (50 or 370 μl) of the cell medium supernatant with CPE were treated with proteinase K and phenol/chloroform extraction. After ethanol precipitation, the DNA samples were analyzed using PCR employing two pairs of primers to amplify insert-specific and viral-genome-specific sequences. The PCR primer targets and their sequences are depicted in FIG. 1. Primers 1, 2, 3 and 4 are represented by SEQ ID NOS:1, 2, 3 and 4, respectively.

As a result, a 1.4 kb cDNA insert and a 0.86 kb viral genome fragment were amplified from the expression vector (positive control) and the DNA samples of the positive cell culture (FIG. 2B, lane 1 and 4, respectively), only the 0.86 kb fragment was amplified from the DNA sample of Ad5/RSV/GL2 virus (negative control, lane 2). No amplified bands appeared from PCR reactions that used either untreated positive cell culture medium supernatant (lane 3).

These results indicated that adenoviruses released into cell culture medium are detectable by PCR, using as little as 50 μL of the cell culture medium supernatant for preparing DNA templates. These results will allow development of a quantitative method for using this technique to determine adenovirus titers, traditionally done by plaque assays.

The wild-type sequence of the p53 cDNA in the Ad5CMV-p53 virus was confirmed by dideoxy DNA sequencing on the CsCl-gradient-purified viral DNA. The control virus Ad5/RSV/GL2, generated in a similar manner, has a structure similar to that of Ad5CMV-p53 except a Rous sarcoma viral promoter and luciferase cDNA were used in its expression cassette. The recombinant adenovirus that carries a *E. coli* β-galactosidase gene (LacZ), Ad5CMV-LacZ, also has a structure similar to that of Ad5CMV-p53, and is obtainable as disclosed in Zhang et al. and from Dr. Frank L. Graham (please see Graham, et al., 1991).

Viral stock, titer, and infection. Individual clones of the Ad5CMV-p53, Ad5/RSV/GL2, and Ad5CMV-LacZ viruses were obtained by plaque-purification according to the method of Graham and Prevec (1991). Single viral clones were propagated in 293 cells. The culture medium of the 293 cells showing the completed cytopathic effect was collected and centrifuged at 1000×g for 10 min. The pooled supernatants were aliquoted and stored at −20° C. as viral stocks. The viral titers were determined by plaque assays (Graham and Prevec, 1991). Infections of the cell lines were carried out by addition of the viral solutions (0.5 ml per 60-mm dish) to cell monolayers and incubation at room temperature for 30 min with brief agitation every 5 min. This was followed by the addition of culture medium and the return of the infected cells to the 37° C. incubator.

The gene transfer efficiency of the recombinant adenoviruses was also evaluated using Ad5CMV-LacZ in a variety of cell lines such as H226Br, H322, H460, HeLa, Hep G2, LM2, and Vero. By X-gal staining, all of the cell lines were stained 97–100% blue after infection with Ad5CMV-LacZ at an MOI of 30 PFU/cell.

EXAMPLE 4

Ad5CMV-p53-Directed p53 Gene Expression in Human Lung Cancer Cells

This example describes the use of recombinant p53 adenovirus to infect human lung cancer cells with a homozygous p53 gene deletion. The results show that growth of these cells and expression of mutant p53 was suppressed, indicating the potential of the Ad5CMV-p53 virion as a useful agent for control of metastatic cells.

Immunohistochemistry was performed on infected cell monolayers that were fixed with 3.8% formalin and treated

with 3% H_2O_2 in methanol for 5 min. Immunohistochemical analysis was performed using Vectastain Elite kit (Vector, Burlingame, Calif.). The primary antibody used was anti-p53 antibody PAb 1801 (Oncogene Science, Manhasset, N.Y.); MOPC-21 (Organon Teknika Corp., West Chester, Pa.) was used as a negative control. The second antibody was an avidin-labeled anti-mouse IgG (Vector). The biotinylated horseradish peroxidase ABC complex reagent was used to detect the antigen-antibody complex. Finally the cells were counterstained with Harris hematoxylin (Sigma) and mounted with Cytoseal 60 (Stephens Scientific, Riverdale, N.J.).

Immunohistochemical analysis of the infected cell lines was performed to examine the in situ expression of p53 expression driven by the CMV promoter of the Ad5CMV-53 virus. In the H358 cell line, which has a homozygous deletion of p53, the p53 gene was transferred with 97–100% efficiency, as detected by immunohistochemical analysis, when the cells were infected with Ad5CMV-p53 at a multiplicity of infection of 30–50 plaque-forming units (PFU)/cell (FIG. 4).

The high transfer efficiency of recombinant adenovirus was confirmed by Ad5CMV-LacZ, a virus which carries the LacZ gene transcribed by the human CMV IE promoter. At an MOI of 30–50 PFU/cell, all of the cells examined, including HeLa, Hep G2, LM2, and the human NSCLC cancer cell lines were 97–100% positive for b-galactosidase activity by X-gal staining. These results indicate that adenoviral vectors are an efficient vehicle for gene transfer into human cancer cells.

Western blotting analysis was performed on total cell lysates prepared by lysing monolayer cells in dishes with SDS-PAGE sample buffer (0.5 ml per 60-mm dish) after rinsing the cells with phosphate-buffered saline (PBS). For SDS-PAGE analysis lanes were loaded with cell lysates equivalent to 5×10^4 cells (10–15 ml). The proteins in the gel were transferred to Hybond™-ECL membrane (Amersham, Arlington Heights, Ill.). The membranes were blocked with 0.5% dry milk in PBS and probed with the primary antibodies: mouse anti-human p53 monoclonal antibody PAb 1801 and mouse anti-human β-actin monoclonal antibody (Amersham), washed and probed with the secondary antibody: horseradish peroxidase-conjugated rabbit anti-mouse IgG (Pierce Chemical Co., Rockford, Ill.). The membranes were developed according to the Amersham's enhanced chemiluminescence protocol. Relative quantities of the exogenous p53 expressed were determined by densitometer (Molecular Dynamics Inc., Sunnyvale, Calif.).

Western blots showed the exogenous p53 protein was expressed at a high level (FIG. 5A lanes 2,3 and 5,6). The protein peaked at day 3 after infection (FIG. 6, insert, 0.5 days to 3 days). As a control, a virion with a structure similar to the recombinant Ad5CMV-p53 of Example 1 was constructed. This virion contains a luciferase cDNA driven by Rous Sarcoma Virus LTR promoter in the expression cassette of the virion. Neither p53 expression nor change in actin expression was detected in the cells infected by the virion Ad5/RSV/GL2.

The recombinant p53 adenovirus was used to infect three human lungs NSCLC cell lines: cell line H358, which has a homozygous deletion of the p53 gene, cell line H322, which has a point mutation of the p53 gene at codon 248 (G to T), and cell line H460, which has a wild-type p53 gene. The growth rate of human NSCLC cells was determined following the inoculation of H322 and H460 (1×10^5) or H358 (2×10^5) in 60-mm culture dishes 24 h before viral infection. The cells were infected with the viruses at a multiplicity of infection (MOI) of 10 PFU/cell. Culture medium was used for the mock infection control. Triplet cultures of each cell line with different treatments were counted daily for days 1–6 after infection.

Growth of the H358 cells infected with Ad5CMV-p53 was greatly inhibited in contrast to that of noninfected cells or the cells infected with the control virion (FIG. 7A). Growth of H322 cells was also greatly inhibited by the p53 virion (FIG. 7B), while that of human lung cancer H460 cells containing wild type p53 was affected to a lesser degree (FIG. 7C). Growth of the AdSCMV-p53 virus-infected H358 cells was inhibited 79%, whereas that of noninfected cells or the cells infected with the control virus were not inhibited. Growth of cell line H322, which has a point mutation in p53, was inhibited 72% by Ad5CMV-p53, while that of cell line H460 containing wild-type p53 was less affected (28% inhibition).

The results indicate that the p53 recombinant adenovirus possesses properties of tumor suppression, working through restoration of the p53 protein function in tumor cells.

EXAMPLE 5

Ad5CMV-p53 in the Treatment of p53 Deficient Cells

The present example concerns the use of recombinant p53 adenovirus to restore growth suppression of tumor cells in vitro and thus to treat the malignant or metastatic growth of cells. It describes some of the ways in which the present invention is envisioned to be of use in the treatment of cancer via adenovirus-mediated gene therapy.

H358 cells were infected with Ad5CMV-p53 and Ad5/RSV/GL2 at a MOI of 10 PFU/cell. An equal amount of cells were treated with medium as a mock infection. Twenty-four hours after infection, the treated cells were harvested and rinsed twice with PBS. For each treatment, three million (3×10^6) cells in a volume of 0.1 ml were injected s.c. to each nude mouse (Harlan Co., Houston, Tex.). Five mice were used for each treatment. Mice were irradiated (300 cGy, ^{60}Co) before injection and examined weekly after injection. Tumor formation was evaluated at the end of a 6-week period and tumor volume was calculated by assuming a spherical shape with the average tumor diameter calculated as the square root of the product of cross-sectional diameters.

To determine the inhibitory effect on tumorigenicity mediated by Ad5CMV-p53 nude mice were injected s.c. with H358 cells (a human NSCLC-type cell) to induce neoplastic growth. Each mouse received one injection of cells that had been infected with Ad5CMV-p53 or Ad5/RSV/GL2 at 10 PFU/cell for 24 h. H358 cells treated with medium alone were used as mock-infected controls. Tumors, first palpable at postinjection day 14, were induced only by the mock- or control virus-infected cells as demonstrated in Table I:

TABLE I

Effect of Ad5CMV-p53 on tumorigenicity of H358 in nude mice[a]

Treatment	No. of Tumors/ No. of Mice (%)	Mean Volume (mm^3 ± SD)
Medium	4/5 (80)	37 ± 12
Ad5/RSV/GL2	3/4 (75)	30 ± 14
Ad5CMV-p53	0/4 (0)	—

[a]The treated H358 cells were injected s.c. at 2×10^6 cells/mouse. Tumor sizes were determined at the end of a 6-week period.

As shown in Table 1 mice that received Ad5CMV-p53-treated cells did not develop tumors. The tumors at the end of a 6-week period were 4–10 mm in diameter. This study was initiated with five mice per group; one mouse each in

the Ad5CMV-p53 or Ad5/RSV/GL2 group failed to complete the study. The early deaths were presumably due to nosocomial infection.

EXAMPLE 6

Ad5CMV-p53 in the Treatment of Lung Cancer

The present example concerns the use of recombinant p53 adenovirus to restore growth suppression of tumor cells in vivo and thus to treat cancers in animals. It describes some of the ways in which the present invention is envisioned to be of use in the treatment of cancer via adenovirus-mediated gene therapy.

The efficacy of Ad5CMV-p53 in inhibiting tumorigenicity was further evaluated in the mouse model of orthotopic human lung cancer. Since H358 and H322 cells did not produce tumors in this model, cell line H226Br was used. This cell line has a squamous lung cancer origin and metastasized from lung to brain. H226br has a point mutation (ATC to GTC) at exon 7, codon 254, of the p53 gene and is tumorigenic in mice.

The procedure for tests in the mouse model of orthotopic human lung cancer has been previously described (Georges, et al., 1993). Briefly, nude mice treated with radiation (300 cGy, ^{60}Co) were inoculated with H226Br cells by intratracheal instillation. Each mouse received 2×10^6 cells in a volume of 0.1 ml PBS. Three days after inoculation, 10 mice per group were treated with 0.1 ml of viruses or vehicle (PBS) by intratracheal instillation once a day for two days. The virus dosage used was 5×10^7 Ad5CMV-p53 or Ad5/RSV/GL2 per mouse. The mice were euthanized at the end of a 6-week period. Tumor formation was evaluated by dissecting the lung and mediastinum tissues and measuring the tumor size. The tumors were confirmed by histologic analysis of the sections of the tumor mass.

The irradiated nude mice were inoculated with 2×10^6 H226Br cells/mouse by intratracheal instillation. Three days after inoculation, each of the mice (8–10 mice per group) were treated with 0.1 ml of either Ad5CMV-p53 or Ad5/RSV/GL2 or vehicle (PBS) by intratracheal instillation once a day for two days. The virus dosage used was 5×10^7 PFU/mouse. Tumor formation was evaluated at the end of a 6-week period by dissecting the lung and mediastinum tissues and measuring the tumor size. A flow chart of the procedure is depicted in FIG. 7, with representative samples of dissection demonstrated in FIG. 8. The detected tumors were confirmed by histologic analysis. The data of tumor measurements are summarized in Table II:

TABLE II

Effect of Ad5CMV-p53 on tumorigenicity of H226Br in mouse model of orthotopic human lung cancer[a]

Treatment	No. mice with Tumors/ Total Mice (%)	Mean Volume (mm^3 ± SD)
Vehicle	7/10 (70)	30 ± 8.4
Ad5/RSV/GL2	8/10 (80)	25 ± 6.9
Ad5CMV-p53	2/8 (25)	8 ± 33[b]

[a]Mice were inoculated with 2×10^6 H226Br cells/mouse intratracheally. On the 3rd day postinoculation, the mice were given either vehicle or viruses (5×10^7 each in 0.1 ml) intratracheally once a day for 2 days. Tumor formation was evaluated at the end of a 6-week period.
[b]$p < 0.05$ by two-way analysis of variance when compared to the groups receiving vehicle (PBS) or virus control.

Only 25% of the Ad5CMV-p53-treated mice formed tumors, whereas in the vehicle or Ad5/RSV/GL2 control group, 70–80% of the treated mice formed tumors. The average tumor size of the Ad5CMV-p53 group was significantly smaller than those of the control groups. These results indicate that Ad5CMV-p53 can prevent H226Br from forming tumors in the mouse model of orthotopic human lung cancer.

EXAMPLE 7

Synergism between p53 and DNA Damage

The biochemical features of programmed cell death (apoptosis) show a characteristic pattern of DNA fragmentation resulting from cleavage of nuclear DNA. Recent studies have demonstrated that induction of apoptosis by chemotherapeutic drugs or ionizing radiation may be related to the status of the p53 gene and that DNA-damaging stimuli are able to elevate intracellular p53 protein levels in cells that are in the process of apoptosis (Lowe, et al., 1993, Clarke, et al., 1993, Fritsche, et al., 1993, Harper, et al., 1993, El-Deiry, et al., 1993). Inhibition of the cell cycle at the G_1 phase by increased levels of the wild-type p53 (wt-p53) protein allows more time for DNA repair; if optimal repair is impossible, p53 may trigger programmed cell death. Thus, p53 may contribute to the induction of apoptotic tumor cell death by chemotherapeutic agents.

Inactivation of the p53 gene by missense mutation or deletion is the most common genetic alteration in human cancers (Levine, et al., 1991, Hollstein, et al., 1991). The loss of p53 function has been reported to enhance cellular resistance to a variety of chemotherapeutic agents (Lowe, et al., 1993). The inventors studies showed that human non-small cell lung cancer (NSCLC) H358 cells, in which both alleles of p53 are deleted, were resistant to chemotherapeutic drugs, whereas cell line WTH226b, which has endogenous wt-p53, readily showed apoptotic cell death 16 hours after treatment with cisplatin (CDDP) and etoposide (VP-16) (T. Fujiwara, E. A. Grimm, T. Mukhopadhyay, J. A. Roth, unpublished data). Therefore, the inventors sought to determine whether the introduction of the wt-p53 gene into H358 cells by an adenoviral vector could increase the cell's sensitivity to the DNA crosslinking agent CDDP in vitro and in vivo.

Materials and Methods

H358 cells were kindly provided by A. Gazdar and J. Minna (Takahashi, et al., 1989).

Adenovirus Vectors

The construction and identification of a recombinant adenovirus vector that contains the CDNA that encodes human wt-p53 (Ad-p53) or luciferase (Ad-Luc) were previously reported (Zhang, et al., 1993). Briefly, the p53 expression cassette that contains human cytomegalovirus promoter, wt-p53 cDNA, and SV40 early polyadenylation signal, was inserted between the XbaI and ClaI sites of pXCJL.1. The p53 shuttle vector and the recombinant plasmid pJM17 were cotransfected into 293 cells (Ad5-transformed human embryonic kidney cell line) by a liposome-mediated technique. The culture supernatant of 293 cells showing .the complete cytopathic effect was collected and used for subsequent infections. The control Ad-Luc virus was generated in a similar manner. Ad-p53 and Ad-Luc viruses were propagated in 293 cells. The presence of replication competent virus was excluded by HeLa cell assays. The viral titers were determined by plaque assays (Graham, et al., 1991).

Detection of Nucleosomal DNA Fragmentation

DNA was isolated from parental, Ad-Luc-infected, and Ad-p53-infected cells that did or did not receive CDDP

treatment, by incubating cells at 55° C. for 6 hours in lysis buffer (50 mM Tris-HCl, pH 8.0, 100 mM EDTA, 100 mM NaCl, 1% SDS, and 50 μg/ml proteinase K). DNA was extracted twice with equal volumes of phenol and once with chloroform-isoamylalcohol (24:1) and then precipitated in ethanol. Samples were subjected to electrophoresis on a 1.5% agarose gel, and visualized by ethidium bromide staining.

TdT-mediated dUTP nick end labeling was performed according to a procedure previously reported (Gavrieli, et al., 1992). Monolayer cells were treated with 0.0% NP-40. The slides were immersed in TdT buffer (30 mM Tris-HCl, pH 7.2; 140 mM sodium cacodylate; 1 mM cobalt chloride) and incubated with biotinylated dUTP (Boehringer Mannheim, Indianapolis, Ind.) and TdT at 370° C. for 45 min. The slides were covered with 2% bovine serum albumin for 10 min and incubated with avidin-biotin complex (Vectastain Elite Kit; Vector Laboratories, Burlingame, Calif.) for 30 min. The colorimetric detection was performed by using diamino-benzidine.

Results

H358 cells were transduced in vitro with the human wt-p53 cDNA by exposure to Ad-p53. Western blot analysis showed a high level of wt-p53 protein expression as early as 24 hours after infection with Ad-p53, but no wt-p53 was detected in parental (uninfected) cells or control cells infected with Ad-Luc (data not shown). Concurrent immunohistochemical evaluation demonstrated detectable wt-p53 protein in more than 80% of infected cells, suggesting that the transfer and expression of p53 by AD-p53 was highly efficient (data not shown).

Continuous exposure of Ad-p53-infected H358 cells to CDDP reduced their viability rapidly, whereas significant cell death for parental and Ad-Luc-infected cells occurred only after 72 hours of exposure to CDDP (FIG. **10**A). Loss of viability was greatly enhanced in cells transduced with Ad-p53. Moreover, the reduction of viability could be observed even when cells were maintained in drug-free medium after 24 hours of exposure, suggesting that lethal damage could be induced within 24 hours (FIG. **10**B). The sensitivity of wt-p53-transduced H358 cells to CDDP was dose dependent (FIG. **10**C).

An internucleosomal DNA ladder indicative of DNA fragmentation was evident in cells expressing wt-p53 after 24 hours of exposure to CDDP; parental and Ad-Luc-infected cells, however, did not show DNA fragmentation (FIG. **11**A). Terminal deoxynucleotidyl transferase (TdT)-mediated 2'-deoxyuridine-5'-triphosphate (dUTP)-biotin nick end labeling, which detects DNA fragmentation characteristic of apoptosis in situ, showed many apoptotic cells in Ad-p53-infected cells treated with CDDP for 24 hours as shown in FIG. **11** G which demonstrates darkly staining nuclei and nuclear fragments not present in FIGS. **11**B–F.

Introduction of wt-p53 is known to induce apoptosis in some types of tumor cell lines with deleted or mutated p53 (Yonish-Rouach, et al., 1991, Shaw, et al., 1992, Ramqvist, et al., 1993). However, overexpression of wt-p53 alone could not promote DNA fragmentation in the p53-negative H358 cell line (FIG. **11**), although their growth was suppressed by Ad-p53 (FIG. **10**). This is compatible with the inventors previous observations showing that stable H358 clones could be obtained after retrovirus-mediated wt-p53 transfer and that the clones grew more slowly than parental cells (Cai, et al., 1993).

The potential therapeutic efficacy of the combination of Ad-p53 and CDDP was evaluated in terms of the relative change in volume of H358 spheroids. The multicellular tumor spheroid model exhibits in vitro a histologic structure similar to that of primary tumors and micrometastases. Treatment with CDDP caused a reduction of relative volume in Ad-p53-infected H358 spheroids, but had no significant effect on parental or Ad-Luc-infected spheroids (FIG. **12**A). In situ TdT-mediated dUTP labeling showed many cells in the process of apoptosis on the surface of Ad-p53-infected spheroids, while no apoptotic cells were seen on spheroids not infected with Ad-p53 (FIG. **12**B–E). The inventors have previously reported that retroviral-mediated wt-p53 expression inhibited growth of H322a spheroids induced by transforming growth factor α (TGF-α) (Fujiwara, et al., 1993). The retroviral vector could not infect H358 spheroids, however, because cells in these spheroids did not proliferate rapidly in response to exogenous TGF-α. The finding that exposure to CDDP reduced the size of H358 spheroids infected with Ad-p53 by inducing apoptosis on the surface suggests that Ad-p53 infects nonproliferating cells and that CDDP initiates the apoptotic process in quiescent cells.

EXAMPLE 8

Using p53 and DNA Damaging Agents in Treatment Regimens

An animal models has been employed as part of preclinical trials, as described hereinbelow and in Examples 5, 6 and 7. Patients for whom the medical indication for adenovirus-mediated gene transfer treatment has been established may be tested for the presence of antibodies directed against adenovirus. If antibodies are present and the patient has a history of allergy to either pharmacological or naturally occurring substances, application of a test dose of on the order of 10^3 to 10^6 recombinant adenovirus under close clinical observation would be indicated.

For the treatment of cancer using Ad5CMV-p53, recombinant adenovirus expressing p53 under the control of suitable promoter/enhancer elements, such as the CMV promoter, would be prepared and purified according to a method that would be acceptable to the Food and Drug Administration (FDA) for administration to human subjects. Such methods include, but are not limited to, cesium chloride density gradient centrifugation, followed by testing for efficacy and purity.

Two basic methods are considered to be suitable for p53 adenovirus treatment methods, a direct or local administration and a more general administration. The present methods are suitable for treating any of the variety of different cancers known to be connected with p53 mutations. In regard to general administration, a simple intravenous injection of adenovirus has been shown to be sufficient to result in viral infection of tissues at sites distant from the injection (Stratford-Perricaudet et al., 1991b), and is thus suitable for the treatment of all p53-linked malignancies. The virus may be administered to patients by means of intravenous administration in any pharmacologically acceptable solution, or as an infusion over a period of time. Generally speaking, it is believed that the effective number of functional virus particles to be administered would range from 1×10^{10} to 5×10^{12}.

Also, particularly where lung cancer is concerned, more direct physical targeting of the recombinant adenovirus could be employed if desired, in an analogous manner to the intratracheal administration of the cystic fibrosis transmembrane conductance regulator (Rosenfeld et al., 1992). This would result in the delivery of recombinant p53 adenovirus closer to the site of the target cells.

Methods

In Situ dUTP labeling with TdT for detection of Apoptosis.

H358 spheroids were fixed on day 3 and stained as described in Example 7. Briefly, labeled TdT probes were contacted to slides immersed in TdT buffer and incubated with biotinylated dUTP and TdT at 37° C. for 45 min. The slides were covered with 2% bovine serum albumin for 10 min and incubated with avidin-biotin complex for 30 min. The calorimetric detection was performed using diaminobenzidine.

Induction of apoptosis by CDDP after in vivo infection with Ad-p53.

H358 cells (5×10^6) in 0.1 ml Hank's balanced salt solution were injected subcutaneously into the right flank of BALB/c female nu/nu mice. Thirty days later, 200 μl of medium alone or medium containing Ad-Luc (10^8 PFU/ml) or Ad-p53 (10^8 PFU/ml) was injected into tumors with a diameter of 5 to 6 mm. Intratumoral injection (100 μl) and peritumoral injection in two opposite sites (50 μl each) were performed. CDDP (3 mg/kg) or control physiological saline was given intraperitoneally. (A) Tumor volume changes. The tumors were measured with calipers in two perpendicular diameters without the knowledge of the treatment groups, and a tumor volume was calculated by assuming a spherical shape with the average tumor diameter calculated as the square root of the product of cross-sectional diameters. Five mice were used for each treatment group and the mean +/− SE is shown. The data was analyzed using the Student's t-test. The arrow shows the day of treatment. Two independent determinations are shown. $p<0.05$ from day 5 in test 1; $p<0.05$ from day 7 in test 2. Histologic study using the TdT-mediated biotin-dUTP labeling technique. Tumors were harvested 5 days after the beginning of treatment and immediately embedded into O. C. T. compound. Frozen tissues were cut in a cryostat at 5-μm thicknesses. The sections were treated with 1 μg/ml proteinase K and stained as described above. All animal care was in accordance with the UT M. D. Anderson Institutional Animal Care and Use Committee.

Results

To demonstrate the in vivo efficacy of the methods and compositions efficacy of a combination of gene replacement therapy and chemotherapy in human cancer, the inventors examined whether sequential administration of Ad-p53 and CDDP could induce apoptosis in vivo. Following 3 days of direct intratumoral injection of Ad-p53 or intraperitoneal administration of CDDP, H358 tumors implanted subcutaneously in nu/nu mice showed a modest slowing of growth. However, if Ad-p53 and CDDP were simultaneously administered, tumors partially regressed and the tumor size remained statistically significantly smaller than those in any of the other treatment groups. The growth inhibitory effect was even more pronounced after two treatment cycles (FIG. 13A). Histologic examination revealed a massive destruction of tumor cells in the area where Ad-p53 was injected in mice treated with CDDP. In situ staining demonstrated many apoptotic cells around acellular spaces (FIG. 13B–E). In contrast, tumors treated with CDDP alone or Ad-p53 alone showed neither acellularity nor apoptotic areas.

In more detail, preferred treatment protocols may be developed along the following lines. Patients may first undergo bronchoscopy to assess the degree of obstruction. As much gross tumor as possible should be resected endoscopically. Patients should preferably undergo bronchoscopy under topical or general anesthesia. A Stifcor™ transbronchial aspiration needle (21 g) will be passed through the biopsy channel of the bronchoscope. The residual tumor site would then be injected with the p53 adenovirus in a small volume such as about 10 ml or less.

In any event, since the adenovirus employed will be replication incompetent, no deleterious effect of the virus itself on subject health is anticipated. However, patients would remain hospitalized during the treatment for at least 48 hours to monitor acute and delayed adverse reactions. Safety-related concerns of the use of replication deficient adenovirus as a gene transfer vehicle in humans have been addressed in the past (Rosenfeld et al., 1992; Jaffe et al., 1992), but the dose of adenovirus to be administered should be appropriately monitored so as to further minimize the chance of untoward side effects.

There are various criteria that one should consider as presenting the existence of a need for response or the existence of toxicity. To assist in determining the existence of toxicity, the tumor bed should be photographed prior to a course of therapy. The longest diameter and its perpendicular will be measured. Size will be reported as the product of the diameters. From these data, one can calculate from these numbers the rate of regrowth of the tumor.

The time to progression can also be measured from the first observation with reduction in tumor bulk until there is evidence of progressive disease. Progressive Disease is defined as an increase of ≧25% in the sum of the products of the diameters of the measured lesion. Patients must have received at least two courses of therapy before a designation of progression is made. The survival of patients will be measured from entry into protocol.

Follow-up examinations would include all those routinely employed in cancer therapy, including monitoring clinical signs and taking biopsies for standard and molecular biological analysis in which the pattern of expression of various p53 genes could be assessed. This would also supply information about the number of cells that have taken up the transferred gene and about the relative promoter strength in vivo. Based on the data obtained adjustments to the treatment may be desirable. These adjustments might include adenovirus constructs that use different promoters or a change in the number of pfu injected to ensure a infection of more, or all, tumor cells without unphysiological overexpression of the recombinant genes.

It is contemplated that the expression of exogenous genes transferred in vivo by adenovirus can persist for extended periods of time. Therapeutically effective long-term expression of virally transferred exogenous genes will have to be addressed on a case by case basis. Marker genes are limited in their usefulness to assess therapeutically relevant persistence of gene expression as the expression levels required for the amelioration of any given genetic disorder might differ considerably from the level required to completely cure another disease.

While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the composition, methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent t those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims. All claimed matter and methods can be made and executed without undue experimentation.

REFERENCES

The following references to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

Bargonetti, et al. (1991) *Cell* 65:1083–1091.

Bishop (1987) *Science* 235:305–311.

Boeheringer Mannheim Biochemicals (1992). DOTAP for high effieiency transfections. *BMBiochemica* 9(1):17.

Boshart, M. et al. (1985). A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. *Cell*, 41:521–530.

Cai, D. W., Mukhopadhyay, T., Liu, T., Fujiwara, T., and Roth, J. A. Stable expression of the wild-type p53 gene in human lung cancer cells after retrovirus-mediated gene transfer. *Human Gene Ther*, 4:617–624, 1993.

Culver, K. W., Ram, Z., Wallbridge, S., Ishii, H., Oldfield, E. H., and Blaese, R. M. In vivo gene transfer with retroviral vector-producer cells for treatment of experimental brain tumors. *Science*, 256:1550–1552, 1992.

Casey, G. Lo-Hueh, M., Lopez, M. E., Vogelstein, B., and Stanbridge, E. J. (1991). Growth suppression of human breast cancer cells by the introduction of a wild-type p53 gene. *Oncogene* 6:1791–1797.

Clarke, A. R., Purdie, C. A., Harrison, D. J., Morris, R. G., Bird, C. C., Hooper, M. L., and Wyllie, A. H. Thymocyte apoptosis induced by p53-dependent and independent pathways. *Nature*, 362: 849–852, 1993.

Dai, et al. (1992) *Proc. Natl. Acad. Sci.* 89:10892–10895.

Doyle, L. A. Mechanisms of drug resistance in human lung cancer cells. *Semin Oncol*, 20:326–337, 1993.

El-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, E., Kinzler, K. W., and Vogelstein, B. WAF1, a potential mediator of p53 tumor suppression. *Cell*, 75:817–825, 1993.

Fritsche, M., Haessler, C., and Brandner, G. Induction of nuclear accumulation of the tumor-suppressor protein p53 by DNA-damaging agents. *Oncogene*, 8:307–318, 1993.

Fields et al. (1990) *Science* 249:1046–1049.

Fujiwara, T., Grimm, E. A., Mukhopadhyay, T., Cai, D. W., Owen-Schaub, L. B., and Roth, J. A. A retroviral wild-type p53 expression vector penetrates human lung cancer spheroids and inhibits growth by inducing apoptosis. *Cancer Res*, 53:4129–4133, 1993.

Gavrieli, Y., Sherman, Y., and Ben-Sasson, S. A. Identification of programmed cell death in situ via specific labelling of nuclear DNA fragmentation. *J Cell Biol*, 119:493–501, 1992.

Georges et al. (1993) *Cancer Res* 53:1743–1746.

Ghosh-Choudhury and Graham (1987) Biochem. *Biophys. Res. Comm.* 147:964–973.

Gluzman et al., (1982) in *Eukaryotic Viral Vectors* (Gluzman, Y., Ed.) pp. 187–192, Cold Spring Harbor Press, Cold Spring Harbor, N.Y.

Graham, F. L. and A. J. van der Eb. (1973). A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* 52:456–467.

Graham, F. L. and Prevec, L. Manipulation of adenovirus vectors.

In: Murray E. J. (ed.), *Methods in Molecular Biology, Gene Transfer and Expression Protocols*, pp. 109–128. New Jersey: The Humana Press Inc, 1991.

Graham, F. L., J. Smiley, W. C. Russell and R. Nairn (1977). Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen Virol.* 36:59–72.

Grunhaus, A. and Horwitx, M. S. (1992). Adenoviruses as cloning vectors. *Semin. Virology* 3:237–2542.

Harper, J. E., Adami, G. R., Wei, N., Keyomarsi, K., and Elledge, S. J. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell*, 75:805–816, 1993.

Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C. (1991). p53 mutations in human cancers. *Science* 253:49–53.

Jaffe et al., (1992) *Nature Genetics* 1:372–378.

Le Gal et al., (1993) *Science* 259:988–990.

Ledley, J. (1987). *J. Pediatrics* 110, 1.

Levine, A. J., Momand, J., and Finlay, C. A. The p53 tumour suppressor gene. *Nature*, 351:453–456, 1991.

Lowe, S. W., Ruley, H. E., Jacks, T., and Housman, D. E. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cell*, 74:957–967, 1993.

Lowe, S. W., Schmitt, E. M., Smith, S. W., Osborne, B. A., and Jacks, T. p53 is required for radiation-induced apoptosis in mouse thymocytes. *Nature*, 362:847–849, 1993.

McGrory, W. J. et al. (1988). A simple technique for the rescue of early region I mutations into infectious human adenovirus type 5. *Virology* 163:614–617.

Mercer, W. E. (1992). Cell cycle regulation and the p53 tumor suppressor protein. *Critic. Rev. Eukar. Gene Express.* 2:251–263.

Mietz, et al. (1992) *EMBO* 11:5013–5020.

Miller 1992. Curr. Top. Microbiol. *Immunol.* 158:1

Montenarh, M. (1992). Biochemical, immunological, and functional aspects of the growth-suppressor/oncoprotein p53. *Critic. Rev. Onco.* 3:233–256.

Mulligan, (1993), *Science* 260:926.

Nicolau, C., et al. (1983). *Proc. Natl. Acad. Sci. U.S.A.* 80, 1068.

Ragot et al., (1993) *Nature*, 361:647–650.

Ramqvist, T., Magnusson, K. P., Wang, Y., Szekeley, L., and Klein, G. Wild-type p53 induces apoptosis in a Burkitt lymphoma (BL) line that carries mutant p53. *Oncogene*, 8: 1495–1500, 1993.

Rosenfeld et al., (1992) *Cell* 68:143–155.

Rosenfeld et al., (1991) *Science*, 232:431–434.

Roth, J., Ruckdeschel, Weisenburger, Editors, *Thoracic Oncology*, First Edition, Chapter 49, pgs 711–721, Sanders, N.Y., 1989.

Shaw, P., Bovey, R., Tardy, S., Sahli, R., Sordat, B., and Costa, J. Induction of apoptosis by wild-type 53 in a human colon tumor-derived cell line. *Proc Natl Acad Sci USA*, 89: 4495–4499, 1992.

Spandidos, et al. (1989), *J. Pathol.*, 157:1–10.

Stewart et al., 1992, *Hum. Gene Ther.* 3:267

Stratford-Perricaudet, L. and M. Perricaudet. (1991a). Gene transfer into animals: the promise of adenovirus. p. 51–61. In O. Cohen-Haguenauer and M. Boiron (Eds.). *Human Gene Transfer*, Editions John Libbey Eurotext, France.

Stratford-Perricaudet et al., (1991b) *Hum. Gene. Ther.* 1:241–256

Takahashi, T., Nau, M. M., Chiba, I., Birrer, M. J., Rosenberg, R. K., Vinocour, M., Levitt, M., Pass, H., Gazdar, A. F., and Minna, J. D. p53: a frequent target for genetic abnormalities in lung cancer. *Science*, 246:491–494, 1989.

Takahashi, T., Carbone, D., Takahashi, T., Nau, M. M., Hida, T., Linnoila, L., Ueda, R., and Minna, J. D. (1992). Wild-type but not mutant p53 suppresses the growth of human lung cancer cells bearing multiple genetic lesions. 1992. *Cancer Res.* 52:2340–2342.

Tishler, R. B., Calderwood, S. K., Coleman, C. N., and Price, B. D. Increases in sequence specific DNA binding by p53

following treatment with chemotherapeutic and DNA damaging agents. *Cancer Res*, 53:2212–2216. 1993.

Tooza. J. (1981). *Molecular biology of DNA Tumor viruses*, 2nd ed. Cold Spring Harbor Laboratory. Cold Spring Harbor. N.Y.

Torchilin et al.. 1992. *Faseb J.* 6:2716

Travali. et al. (1990). *FASEB*, 4:3209–3214.

Weinberg. R. A. (1991). *Tumor suppressor gene. Science* 254:1138–1145.

Wilcock. et al. (1991) *Nature* 349:429–431.

Wolff. J. A.. Malone. R. W.. Williams. P.. Chong. W.. Acsadi. G.. Jani. A.. and Felgner. P. L. (1990) Direct gene transfer into mouse muscle in vivo. *Science* 247.1465–1468.

Yonish-Rouach. E.. Resnitzky. D.. Rotem. J.. Sachs. L.. Kimchi. A.. and Oren. M. Wild-type p53 induces apoptosis of myeloid leukemic cells that is inhibited by interleukin-6. *Nature*, 352:345–347. 1991.

Zakut-Houri et al. (1985). *EMBO J.*, 4:1251–1255.

Zhan. Q.. Carrier. F.. and Fornace Jr.. A. J. Induction of cellular p53 activity by DNA-damaging agents and growth arrest. *Mol Cell Biol*, 13: 4242–4250. 1993.

Zhang. W. W.. Fang. X.. Branch. C. D.. Mazur. W.. French. B. A.. and Roth. J. A. Generation and identification of recombinant adenovirus by lipsome-mediated transfection and PCR analysis. *BioTechniques*, 1993.

Zhang. W. W.. Fang. X.. Mazur. W.. French. B. A.. Georges. R. N.. and Roth. J. A. High-efficiency gene transfer and high-level expression of wild-type p53 in human lung cancer cells mediated by recombinant adenovirus. *Cancer Gene Therapy*, 1993.

Zhu. et al.. 1993. *Science* 261:209–211.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i i i) NUMBER OF SEQUENCES: 4

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GGCCCACCCC CTTGGCTTC 19

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TTGTAACCAT TATAAGCTGC 20

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TCGTTTCTCA GCAGCTGTTG 20

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

-continued

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CATCTGAACT CAAAGCGTGG 20

What is claimed is:

1. A method of killing a tumor cell in a patient in need thereof, comprising directly administering to said tumor cell therapeutically effective amounts of a viral vector and a DNA damaging agent, wherein said viral vector comprises a DNA sequec encoding p53 operatively linked to a promoter, and wherein expression of said p53 and DNA damage result in the killing of said tumor cell.

2. The method of claim 1, wherein said viral vector is selected from the group consisting of retrovirus, adenovirus, herpesvirus, adeno-associated virus and cytomegalovirus.

3. The method claim 2, wherein the tumor cell is contacted with a pharmaceutical composition comprising a DNA damaging compound.

4. The method of claim 3, wherein the DNA damaging agent is cisplatin.

5. The method of claim 4, wherein said cisplatin is administered at 20 mg/m^2.

6. The method of claim 3, wherein the DNA damaging agent is doxorubicin.

7. The method of claim 6, wherein said doxorabicin is administered at 25–75 mg/m^2.

8. The method of claim 3, wherein the DNA damaging agent is etoposide.

9. The method of claim 8, wherein said etoposide is administered at 35–50 mg/m^2.

10. The method of claim 3, wherein the DNA damaging agent is verapamil.

11. The method of claim 3, wherein the DNA damaging agent is podophyllotoxin.

12. The method of claim 3, wherein the DNA damaging agent is 5-FU.

13. The method of claim 12, wherein said 5-FU is administered at 3–15 mg/kg.

14. The method of claim 2, wherein said viral vector is a retroviral vector.

15. The method of claim 2, wherein said viral vector is an adenoviral vector.

16. The method of claim 15, wherein the amount of adenoviral vector is 1×10^5 to 1×10^{12} pfu.

17. The method of claim 16, wherein said amount is 5×10^7 pfu.

18. The method of claim 16, wherein said amount is 2×10^7 pfu.

19. The method of claim 2, wherein said viral vector is a herpesviral vector.

20. The method of claim 2, wherein said viral vector is an adeno-associated viral vector.

21. The method of claim 2, wherein said viral vector is a cytomegaloviral vector.

22. The method of claim 1, wherein said promoter is a constitutives promoter.

23. The method of claim 22, wherein the promoter is selected from the group consisting of SV40, CMV and RSV.

24. The method of claim 23, wherein the promoter is the CMV IE promoter.

25. The method of claim 24, wherein the viral vector further comprises a polyadenylation signal.

26. The method of claim 25, wherein the viral vector is an adenoviral vector.

27. The method of claim 1, wherein the tumor cell is contacted with a DNA damaging agent by irradiating the tumor cell with X-ray radiation, UV-irradiation, γ-irradiation or microwaves.

28. The method of claim 27, wherein the tumor cell is contacted with a DNA damaging agent by irradiating the tumor cell with X-ray radiation.

29. The method of claim 28, wherein the x-ray dosage is between 2000 and 6000 roentgens.

30. The method of claim 28, wherein the x-ray -dosage is between 50 and 200 roentgens.

31. The method of claim 27, wherein the tumor cell is contacted with a DNA damaging agent by irradiating the tumor cell with UV-irradiation.

32. The method of claim 27, wherein the tumor cell is contacted with a DNA damaging agent by irradiating the tumor cell with γ-irradiation.

33. The method of claim 27, wherein the tumor cell is contacted with a DNA damaging agent by irradiating the tumor cell with microwaves.

34. The method claim 1, wherein the tumor cell is contacted with a DNA damaging agent by administering to the patient a pharmaceutical composition comprising a DNA damaging compound.

35. The method of claim 1, wherein said viral vector is administered prior to said DNA damaging agent.

36. The method of claim 1, wherein said viral vector is administered after said DNA damaging agent.

37. The method of claim 1, wherein said viral vector is administered at the same time as said DNA damaging agent.

38. The method of claim 1, wherein said viral vector is delivered endoscopically, intravenously, intratracheally, intralesionally, percutaneously or subcutaneously.

39. The method of claim 1, wherein said tumor is located in a resected tumor bed.

40. The method of claim 1, wherein said administering is repeated.

41. The method of claim 1, wherein said tumor cell is a lung cancer cell.

42. The method of claim 41, wherein said lung cancer cell is non-small cell lung carcinoma cell.

43. The method of claim 42, wherein said non-small cell lung carcinoma cell is a sqamous carcinoma cell.

44. The method of claim 42, wherein said non-small cell lung carcinoma cell is an adenocarcinoma cell.

45. The method of claim 42, wherein said non-small cell lung carcinoma cell is a large-cell undifferentiated carcinoma cell.

46. The method of claim 41, wherein said lung cancer cell is a small cell lung carcinoma cell.

47. The method of claim 1, wherein said tumor cell is an epithelial tumor cell.

48. The method of claim 1, wherein said tumor cell is a breast cancer cell.

49. The method of claim 1, wherein said viral vector is administered in about 0.1 ml.

50. The method of claim 1, wherein said viral vector is administered in about 10 ml.

51. A method of treating cancer in a cancer patient, comprising directly administering to a tumor site therapeutically effective amounts of a viral vector and a DNA damaging agent, wherein said viral vector comprises a DNA sequence encoding p53 operatively linked to a promoter, and wherein expression of said p53 and DNA damage result in treatment of said cancer.

52. The method of claim 51, wherein said viral vector is selected from the group consisting of retrovirus, adenovirus, herpesvirus, adeno-associated virus and cytomegalovirus.

53. The method of claim 52, wherein said viral vector is a retroviral vector.

54. The method of claim 52, wherein said viral vector is an adenoviral vector.

55. The method of claim 54, wherein the amount of adenoviral vector is 1×10^5 to 1×10^{12} pfu.

56. The method of claim 55, wherein said amount is 5×10^7 pfu.

57. The method of claim 55, wherein said amount is 2×10^7 pfu.

58. The method of claim 52, wherein said viral vector is a herpesviral vector.

59. The method of claim 52, wherein said viral vector is an adeno-associated viral vector.

60. The method of claim 52, wherein said viral vector is a cytomegaloviral vector.

61. The method of claim 51, wherein the tumor site is contacted with a DNA damaging agent by irradiating the tumor site with X-ray radiation, UV-irradiation, γ-irradiation or microwaves.

62. The method of claim 61, wherein the tumor site is contacted with a DNA damaging agent by irradiating the tumor site with X-ray radiation.

63. The method of claim 62, wherein the x-ray dosage is between 2000 and 6000 roentgens.

64. The method of claim 62, wherein the x-ray dosage is between 50 and 200 roentgens.

65. The method of claim 61, wherein the tumor site is contacted with a DNA damaging agent by irradiating the tumor site with UV-irradiation.

66. The method of claim 61, wherein the tumor site is contacted with a DNA damaging agent by irradiating the tumor site with γ-irradiation.

67. The method of claim 61, wherein the tumor site is contacted with a DNA damaging agent by irradiating the tumor site with microwaves.

68. The method claim 51, wherein the tumor site is contacted with a DNA damaging agent by admninistering to the patient a a pharmaeutical composition comprising a DNA damaging compound.

69. The method of claim 68, wherein the DNA damaging compound is cisplatin.

70. The method of claim 69, wherein said cisplatin is administered at 20 mg/m^2.

71. The method of claim 68, wherein the DNA damaging agent is doxorubicin.

72. The method of claim 71, wherein said etoposide is administered at 35–50 mg/m^2.

73. The method of claim 72, wherein said doxorubicin is administered at 25–75mg/m^2.

74. The method of claim 68, wherein the DNA damaging agent is etoposide.

75. The method of claim 68, wherein the DNA damaging agent is verapamil.

76. The method of claim 68, wherein the DNA damaging agent is podophyllotoxin.

77. The method of claim 68, wherein the DNA damaging agent is 5-FU.

78. The method of claim 77, wherein said 5-FU is administered at 3–15 mg/kg.

79. The method of claim 51, wherein said viral vector is administered prior to said DNA damaging agent.

80. The method of claim 79, wherein the period between administration of the viral vector and DNA damaging agent is between 12 and 24 hours.

81. The method of claim 79, wherein the period between administration of the viral vector and DNA damaging agent is between 6 and 12 hours.

82. The method of claim 79, wherein the period between administration of the viral vector and DNA damaging agent is about 12 hours.

83. The method of claim 51, wherein said viral vector is administered after said DNA damaging agent.

84. The method of claim 83, wherein the period between administration of the DNA damaging agent and viral vector is between 12 and 24 hours.

85. The method of claim 83, wherein the period between administration of the DNA damaging agent and viral vector is between 6 and 12hours.

86. The method of claim 83, wherein the period between administration of the DNA damaging agent and viral vector is about 12 hours.

87. The method of claim 51, wherein said viral vector is administered at the same time as said DNA damaging agent.

88. The method of claim 51, wherein said viral vector is delivered endoscopically, intravenously, intratracheally, intralesionally, percutaneously or subcutaneously.

89. The method of claim 51, wherein said tumor site is a resected tumor bed.

90. The method of claim 51, wherein said administration is repeated.

91. The method of claim 51, wherein said cancer is a lung cancer.

92. The method of claim 91, wherein said lung cancer is a non-small cell lung carcinoma cancer.

93. The method of claim 92, wherein said non-small cell lung carcinoma cancer is a squamous carcinoma cancer.

94. The method of claim 92, wherein said non-small cell lung carcinoma cancer is an adenocarcinoma cancer.

95. The method of claim 92, wherein said non-small cell lung carcinoma cancer is a large-cell undifferentiated carcinoma cancer.

96. The method of claim 91, wherein said lung cancer is a small cell lung carcinoma cancer.

97. The method of claim 51, wherein said cancer is an epithelial cancer.

98. The method of claim 51, wherein said cancer is breast cancer.

99. The method of claim 51, wherein said viral vector is administered in about 0.1 ml.

100. The method of claim 51, wherein said viral vector is administered in about 10 ml.

101. The method of claim 2, wherein said promoter is a constitutives promoter.

102. The method of claim 101, wherein said promoter is selected from the group consisting of SV40, CMV and RSV.

103. The method of claim 102, wherein the promoter is the CMV IE promoter.

104. The method of claim 103, wherein the viral vector further comprises a polyadenylation signal.

105. The method of claim 104, wherein the viral vector is an adenoviral vector.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,747,469

DATED : May 5, 1998

INVENTOR(S) : Roth et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Page 3, item [56], insert --Petty et al., "Expression of the p53 tumour suppressor gene product is a determinant of chemosensitivity," Journal of Cancer Research and Clinical Oncology, 120:R108, 1994.--

In claim 1, column 37, line 15, delete "sequec" and insert --sequence-- therefor.

In claim 7, column 37, line 30, delete "doxorabicin" and insert --doxorubicin-- therefor.

In claim 22, column 37, line 61, delete "constitutives" and insert --constitutive-- therefor.

In claim 30, column 38, line 21, delete "-dosage" and insert --dosage-- therefor.

In claim 43, column 38, line 54, delete "sqamous" and insert --squamous-- therefor.

In claim 68, column 39, line 50, delete "a a" and insert --a-- therefor.

In claim 73, column 39, line 61, delete "25-75mg/m^2" and insert --25-75 mg/m^2-- therefor.

In claim 85, column 40, line 23, delete "12hours" and insert --12 hours-- therefor.

In claim 101, column 40, line 57, delete "2" and insert --51-- therefor.

In claim 101, column 40, line 58, delete "constitutives" and insert --constitutive-- therefor.

Signed and Sealed this

Twenty-seventh Day of June, 2000

Attest:

Q. TODD DICKINSON

Attesting Officer

Director of Patents and Trademarks

Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 237–241, January 1997
Medical Sciences

Terminal differentiation of human liposarcoma cells induced by ligands for peroxisome proliferator-activated receptor γ and the retinoid X receptor

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ABSTRACT **Induction of terminal differentiation represents a promising therapeutic approach to certain human malignancies. The peroxisome proliferator-activated receptor γ (PPARγ) and the retinoid X receptor α (RXRα) form a heterodimeric complex that functions as a central regulator of adipocyte differentiation. Natural and synthetic ligands for both receptors have been identified. We demonstrate here that PPARγ is expressed at high levels in each of the major histologic types of human liposarcoma. Moreover, primary human liposarcoma cells can be induced to undergo terminal differentiation by treatment with the PPARγ ligand pioglitazone, suggesting that the differentiation block in these cells can be overcome by maximal activation of the PPAR pathway. We further demonstrate that RXR-specific ligands are also potent adipogenic agents in cells expressing the PPARγ/RXRα heterodimer, and that simultaneous treatment of liposarcoma cells with both PPARγ- and RXR-specific ligands results in an additive stimulation of differentiation. Liposarcoma cell differentiation is characterized by accumulation of intracellular lipid, induction of adipocyte-specific genes, and withdrawal from the cell cycle. These results suggest that PPARγ ligands such as thiazolidinediones and RXR-specific retinoids may be useful therapeutic agents for the treatment of liposarcoma.**

Liposarcoma is the most common soft tissue malignancy in adults, accounting for at least 20% of all sarcomas in this age group (1). Multiple histologic subtypes of liposarcoma are recognized, including well differentiated, dedifferentiated, myxoid, round cell, and pleomorphic. The histologic subtype is predictive of both the clinical course of the disease and the ultimate prognosis (2). Localized disease is treated primarily with surgery, often in combination with radiotherapy. Metastatic liposarcoma is associated with an extremely poor prognosis, with average 5-year survivals ranging from 70% to 25% depending on the type of tumor. Conventional chemotherapy for metastatic liposarcoma leads to complete response in only about 10% of cases, and thus for most patients is largely palliative (3, 4).

Induction of terminal differentiation represents a promising alternative to conventional chemotherapy for certain malignancies. For example, the retinoic acid receptor α, which plays an important role in the differentiation and malignant transformation of cells of the myelocytic lineage, has been used as a target for intervention in acute promyelocytic leukemia (5, 6). Differentiation therapy with all-trans retinoic acid has become the standard of care for this disease. Nuclear receptors that regulate growth and differentiation of other cell types may also represent potential targets for differentiation therapy.

The nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ) plays a central role in the process of adipocyte differentiation. This receptor and its heterodimeric partner retinoid X receptor α (RXRα) form a DNA-binding complex that regulates transcription of adipocyte-specific genes (7–11). Expression and activation of PPARγ in fibroblastic cells triggers the adipocyte gene expression cascade and leads to the development of the adipose phenotype (12). The thiazolidinedione class of antidiabetic drugs and the nuclear prostanoid 15-deoxy-Δ12,14-prostaglandin J2 have recently been identified as ligands for PPARγ (13–15). Members of the C/EBP family of transcription factors have also been shown to promote adipocyte differentiation (16, 17), and recent evidence suggests that the adipogenic activity of some C/EBP family members is at least in part related to their ability to induce PPARγ expression (18).

All-trans retinoic acid is known to be an effective inhibitor of adipocyte differentiation (19, 20). Experiments using receptor-specific agonists have indicated that this effect is mediated primarily by the retinoic acid receptor α. The PPARγ partner RXRα, which binds 9-cis retinoic acid but not all-trans retinoic acid, responds to a distinct retinoid signaling pathway (21, 22). While studies have suggested that the transcriptional activity of the PPAR/RXR heterodimer is maximal in the presence of both PPAR and RXR activators (7, 8), it is not known how the binding of an RXR ligand modulates the adipogenic activity of the PPARγ/RXRα complex.

We report here that PPARγ is expressed consistently in each of the major histologic types of human liposarcoma, and that primary human liposarcoma cells can be induced to undergo terminal differentiation *in vitro* by treatment with thiazolidinediones and RXR-specific retinoids. Our results suggest that these compounds may be useful as differentiation therapy for liposarcoma.

MATERIALS AND METHODS

Tissue Samples and Cytogenetics. Normal human tissues, liposarcomas, and other soft tissue sarcomas were obtained from surgical cases at the Brigham and Women's Hospital (Boston). All tissue samples were taken from homogeneous and viable portions of the resected sample by the pathologist

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Abbreviations: PPARγ, peroxisome proliferator-activated receptor γ; RXRα, retinoid X receptor α.

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and frozen within 10 min of excision. Hematoxylin- and eosin-stained sections of each soft tissue sarcoma were reviewed by a single pathologist (C.D.M.F.) and classified according to histologic type and grade. Histologic classification was based solely on morphologic pattern recognition using conventional diagnostic criteria. For cytogenetic analysis tumors were disaggregated with collagenase and harvested after 3–7 days of culture in T25 flasks (23). Metaphase cells were analyzed by trypsin–Giemsa (24) and quinacrine mustard banding (25).

Cell Culture and RNA Analysis. Primary liposarcoma cells were isolated from selected freshly harvested tumors as described (refs. 3 and 25, and references therein). Primary cells were cultured in 60-mm dishes in RPMI 1640 medium containing 15% Cosmic Calf Serum (HyClone), 20 μg/ml bovine pituitary extract (Collaborative Research), and 5 μg/ml insulin. Pioglitazone (Upjohn), troglitazone (Parke–Davis/Warner–Lambert), BRL49653 (Biomol, Plymouth Meeting, PA), and LG268 (Ligand Pharmaceuticals, La Jolla, CA) were dissolved in dimethyl sulfoxide and applied to cells in less than 0.1% of the media volume. The NIH PPARγ and NIH vector cells were derived by retroviral infection and cultured as described (12). Differentiated cells were fixed and stained for neutral lipid with oil red O (26). BrdUrd labeling was performed using the 5-bromo-2′-deoxyuridine Labeling and Detection Kit II (Boehringer Mannheim), according to the manufacturers' instructions. RNA isolation and Northern blot analysis was carried out as described (8).

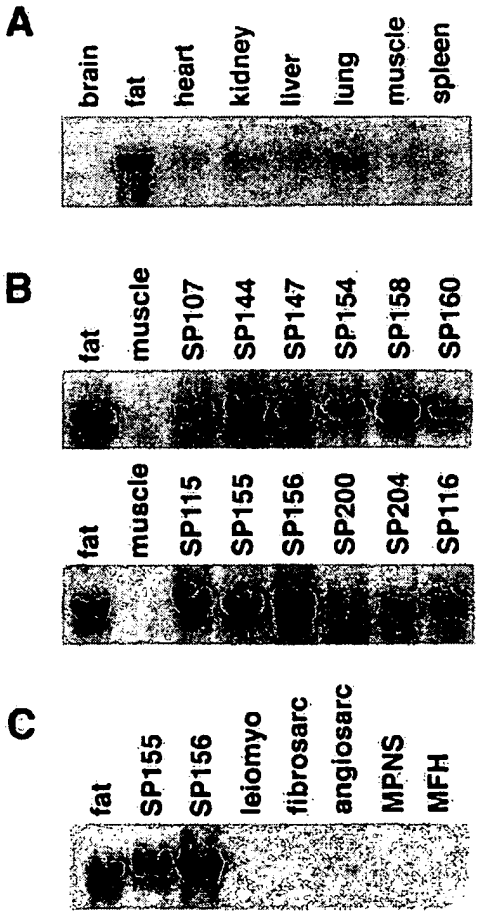

FIG. 1. Expression of PPARγ mRNA in human tissues (*A*), human liposarcomas (*B*), and other soft tissue sarcomas (*C*). Total RNA (15 μg per lane) was isolated from human tumors, electrophoresed through formaldehyde-containing agarose gels, blotted to nylon, and hybridized with ^{32}P-labeled hPPARγ cDNA. Equivalent amounts of intact RNA was run in each lane as indicated by hybridization to a 36B4 cDNA probe (not shown). MPNS, malignant peripheral nerve sheath tumor; MFH, malignant fibrous histiocytoma.

RESULTS

PPARγ is expressed at high levels in the adipose tissues of mouse and rat (8, 27). To determine the tissue distribution of this receptor in humans, we performed Northern blot analysis of RNA prepared from a variety of human tissues. As shown in Fig. 1*A*, human PPARγ is expressed at predominantly in adipose tissue and at lower levels in several other tissues.

Tumorigenesis frequently involves the inactivation or down-regulation of genes responsible for initiating and maintaining a differentiated phenotype. As PPARγ appears to play a central role in the adipocyte differentiation process, we examined the expression of this receptor in a series of human liposarcomas. This series included RNA prepared from each of the three major histologic subtypes of liposarcoma: well differentiated/dedifferentiated, myxoid/round cell, and pleomorphic. The histologic and cytogenetic characteristics of each tumor are given in Table 1. For the most part, the well differentiated/dedifferentiated tumors exhibited ring chromosomes and giant marker chromosomes, the myxoid/round cell liposarcomas exhibited the characteristic t(12;16)(q13p11) translocation, and the pleomorphic forms exhibited complex rearrangements (28–30). Surprisingly, despite their block in differentiation, each liposarcoma examined was found to express significant levels of PPARγ RNA, comparable to that of normal fat (Fig. 1*B*). These results suggest that most if not all liposarcomas have been transformed at a point in the differentiation process after induction of PPARγ expression. In contrast, PPARγ RNA was not expressed at significant levels in any other type of soft tissue sarcoma examined

Table 1. Histologic and cytogenetic characteristics of human liposarcomas

Tumor	Histology	Cytogenetics	Cell culture
107SP	Well differentiated	47-48,XX,+1-2mars	NA
115SP	High grade myxoid/round cell	80-91,XXXX,t(12;16)(q13;p11)x2	NA
116SP	High grade, pleomorphic, and myxoid areas	ND	NA
200SP	High grade, mixed pleomorphic/round cell	ND	NA
203SP	Well differentiated	48-50,XY,del(16)(q36),+2-4 r	LS175
204SP	Well differentiated	48,XX,add(7)(q36),del(11)(p13),+2 mars	LS857
P144	Well differentiated	48,XX,+2r	NA
P147	Well differentiated, lipoma-like, sclerosing	46-49,XX,add(9)(q34),+1-2r, +1-2 mars	NA
P154	Atypical lipoma/well differentiated liposarc	ND	NA
P155	Intermediate grade, myxoid > round cell	46,XY,t(12;16)(q13;p11)	LS707
P156	Intermediate grade, round cell > myxoid	49XY,+del(1)(p32),+2,+8,t(12;16)(q13;p11)	NA
P158	Well differentiated	ND	NA
P160	Well differentiated with dedifferentiated areas	43-49,XX,add(1)(q43),−11,−13,−13,+1-3 r	NA

ND, not determined; NA, not available

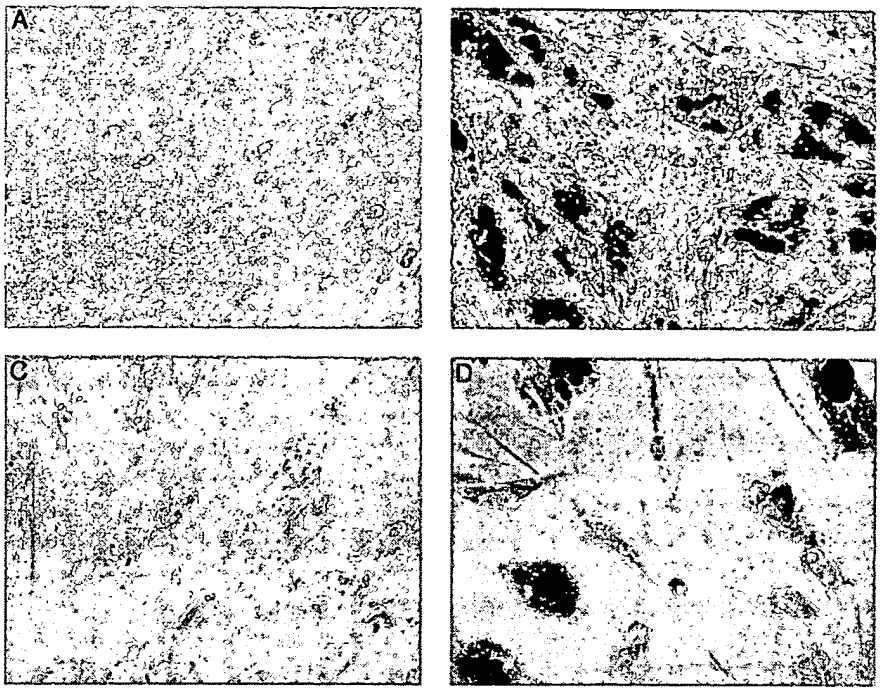

FIG. 2. Pioglitazone induces differentiation of cultured human liposarcoma cells. Primary LS857 (*A* and *B*) and LS707 (*C* and *D*) cells were isolated and cultured in 60-mm dishes. At confluence, cells were cultured for 7 days in the presence (*B* and *D*) or absence (*A* and *C*) of 10 μM pioglitazone. After an additional 4 days of culture, cells were fixed and stained with oil red O. (×40.)

including leiomyosarcoma (n = 4), fibrosarcoma (n = 1), angiosarcoma (n = 1), malignant peripheral nerve sheath tumor (n = 1), or malignant fibrous histiocytoma (n = 1) (Fig. 1*C*). Thus, PPARγ may be a sensitive marker for distinguishing liposarcoma from other histologic types of soft tissue sarcoma.

The thiazolidinedione antidiabetic drugs have recently been identified as ligand activators of murine PPARγ (13, 15). In data not shown, we have found that the thiazolidinediones are also effective activators of human PPARγ, and their relative potency parallels their potency as insulin sensitizing agents *in vivo*: BRL49653 > troglitazone > pioglitazone. These results are consistent with the hypothesis that PPARγ mediates the antidiabetic action of these compounds in humans.

Liposarcomas have presumably acquired one or more genetic defects that interfere with the course of normal adipocyte development. The observation that PPARγ is expressed consistently in these tumors raised the possibility that the malignant cells might be forced to complete the differentiation program by maximally activating the PPARγ pathway with thiazolidinedione ligand. To address this possibility, primary cells isolated from three human liposarcomas were cultured *in vitro*. Primary cell strains LS857 and LS175 were derived from well differentiated liposarcomas and LS707 was derived from an intermediate grade myxoid/round cell liposarcoma (Table 1). High grade pleomorphic liposarcoma cells could not be expanded to sufficient numbers to permit studies of differentiation. Cytogenetic analysis confirmed that the karyotype of the cells in each culture was characteristic of the parent liposarcoma.

When cultured in the presence of fetal bovine serum and insulin, conditions permissive for adipocyte differentiation, all three cell strains maintain a fibroblastic morphology. When cultures were treated for 7 days with 10 μM of the PPARγ ligand pioglitazone, the cells readily accumulated lipid and adopted a morphology characteristic of mature cultured adipocytes (Fig. 2 and data not shown). The degree of morphologically recognizable differentiation varied from 40% in the LS857 cells to 75% in the LS175 cells. After induction for 7 days with thiazolidinedione, cells maintained their differentiated morphology even when pioglitazone was withdrawn. This experiment was performed at least twice with each cell strain with similar results. No differentiation was observed with cultured primary leiomyosarcoma cells, which do not express PPARγ (not shown).

Previous work has suggested that maximal transcriptional activity of the PPAR/RXR heterodimer is achieved when both receptors are bound by their respective ligands (7, 8, 27). We hypothesized that simultaneous exposure of competent cells to both PPARγ and RXR-specific ligands might provide a stronger adipogenic signal than a PPARγ ligand alone. The ability of the RXR-specific ligand LG268 to promote adipocyte differentiation was investigated using NIH 3T3 fibroblasts that express PPARγ from a retroviral vector (12). We have previously shown that wild-type NIH 3T3 cells express RXRα but not PPARγ. As shown in Table 2, treatment of confluent NIH PPARγ cells for 7 days with 50 nM LG268 resulted in significant stimulation of adipocyte differentiation, comparable to that seen with 7 days of treatment with 1 μM pioglitazone alone. Simultaneous exposure to both activators resulted in an additive effect. LG268 had no effect on NIH vector cells, indicating that the adipogenic activity of this compound, like that of pioglitazone, is dependent on the presence of PPARγ. Similar results were obtained with the preadipocyte cell lines

Table 2. Thiazolidinedione and RXR-specific retinoids stimulate differentiation of PPARγ-expressing fibroblasts

	% lipid containing cells			
Cell line	No activator	+PIO	+LG268	+PIO+LG268
NIH vector	0	0	0	<1
NIH PPARγ	2-5	55-75	50-65	>90

NIH vector and NIH PPARγ cells were treated at confluence for 7 days with no activator, 1 μM pioglitazone alone, 50 nM LG268 alone, or 5 μM thiazolidinedione and 50 nM LG268 as indicated. After an additional 4 days of culture cells were fixed and stained with oil red O. Data are presented as the range of morphologically recognizable differentiation observed for each cell line over three separate experiments.

3T3-L1 and 3T3-F442A, which express both PPARγ and RXRα (data not shown). Northern blot analysis confirmed that pioglitazone and LG268 had an additive effect on the induction of the adipocyte-specific genes aP2 and adipsin in NIH PPARγ cells (Fig. 3).

We next examined the ability of LG268 to promote differentiation of human liposarcoma cells. As shown in Fig. 4, treatment of LS857 cells for 7 days with 100 nM LG268 led to a significant degree of adipocyte differentiation, similar to that seen with 10 μM pioglitazone alone. When LS857 cells were treated simultaneously with LG268 and either pioglitazone or BRL49653, an additive effect on differentiation was observed. Expression of adipocyte-specific markers in these cells was confirmed by Northern blotting (Fig. 3). LS857 cells, like the tumor from which they were derived, express PPARγ mRNA (c.f., Fig. 1*B*, tumor 204SP). Treatment of LS857 cells with pioglitazone leads to the induction of the mRNAs encoding aP2 and adipsin (Fig. 3). Simultaneous treatment with pioglitazone and LG268 results in an additive effect. In summary, treatment of LS857 cells with thiazolidinediones and RXR-specific retinoids leads to changes in morphology and gene expression consistent with terminal adipocyte differentiation.

Terminal differentiation of white adipocytes *in vitro* and *in vivo* is characterized by permanent withdrawal from the cell cycle. A critical question is whether thiazolidinedione-induced differentiation of liposarcoma cells is accompanied by growth arrest. To address this issue, LS857 cells were cultured in the presence or absence of pioglitazone. Following induction of morphologic differentiation, pioglitazone was withdrawn. After 48 hr of continued culture in the absence of pioglitazone, cells were labeled for 48 hr with BrdUrd. In the two experiments presented in Table 3, 26–34% of the cells contained visible cytoplasmic lipid, and 40–51% of the cells in this culture stained positive for BrdUrd incorporation by light microscopy. However, of those cells containing lipid, only 3–4% stain positive for BrdUrd. These results demonstrate that thiazolidinedione-induced differentiation of LS857 cells leads to cell cycle withdrawal.

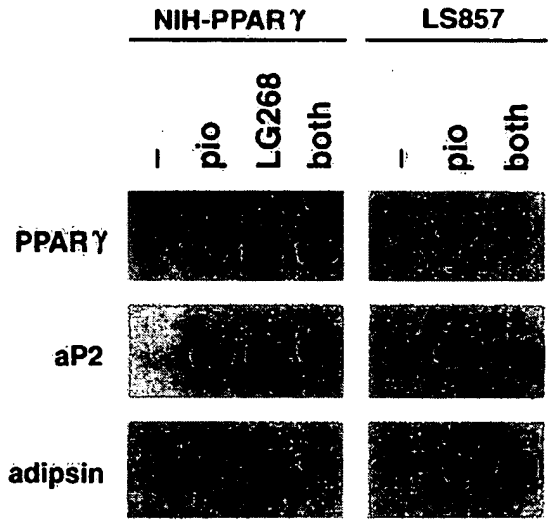

FIG. 3. PPARγ- and RXR-specific ligands induce expression of markers of terminal adipocyte differentiation in PPARγ-expressing fibroblasts and human liposarcoma cells. NIH PPARγ and LS857 cells were treated for 7 days with no activator, pioglitazone alone, LG268 alone, or pioglitazone and LG268 as indicated (both). Total RNA (10 μg per lane) was blotted to nylon and hybridized with ^{32}P-labeled human or murine PPARγ, aP2, and adipsin cDNA. Equivalent amounts of intact RNA were run in each lane as indicated by hybridization to a 36B4 cDNA probe (not shown). Two PPARγ transcripts are present in NIH PPARγ cells, the viral transcript (*Upper*) and the endogenous transcript (*Lower*).

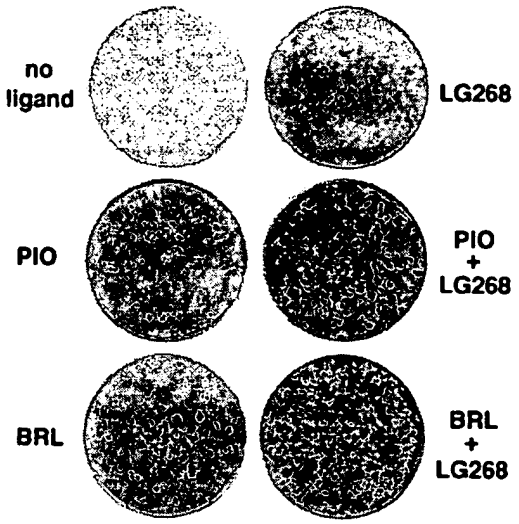

FIG. 4. Induction of differentiation in human liposarcoma cells by thiazolidinediones and RXR-specific retinoids. LS857 cells were treated at confluence for 7 days with no activator, 5 μM thiazolidinedione (BRL 49653 or pioglitazone), 50 nM LG268, or both as indicated. After an additional 4 days of culture cells were fixed and stained with oil red O. Macroscopic view of the 60-mM culture dishes is shown.

DISCUSSION

Terminal differentiation of most specialized cell types, including white adipocytes, is linked to cell cycle withdrawal. Tumorigenesis is characterized by a loss of cell cycle control and a concordant block in the differentiation program. We have demonstrated here that most human liposarcomas express high levels of the adipocyte regulatory complex PPARγ/RXRα and that PPARγ- and RXRα-specific ligands are able to trigger terminal differentiation of primary human liposarcoma cells *in vitro*. These results suggest that the developmental defect in most liposarcomas is downstream of PPARγ expression, and that in at least some tumor cells this developmental block can be overcome by maximal activation of the PPARγ pathway.

While the precise nature of the developmental defects in liposarcoma is not yet clear, it is likely these defects ultimately lead to the inactivation or antagonism of one or more adipocyte transcriptional regulatory proteins. Members of both the C/EBP and PPAR transcription factor families have been shown to play central and complementary roles in adipogenesis in murine models (16, 18, 31–33). Interestingly, the C/EBP family has previously been implicated in the pathogenesis of human myxoid liposarcoma through the characterization of the t(12;16) translocation associated with this tumor. This rearrangement fuses the gene for the C/EBP family member CHOP on chromosome 12 to that of the RNA-binding protein TLS on chromosome 16 (34). The precise mechanism whereby

Table 3. Differentiation of LS857 cells by thiazolidinediones and RXR-specific retinoids leads to cell cycle withdrawal

	Control	PIO/268 1	PIO/268 2
Cells counted	500	510	595
BrdUrd + (%)	232 (46)	173 (34)	156 (26)
Lipid + (%)	0	204 (40)	233 (51)
BrdUrd + lipid + (%)	NA	22 (4)	17 (3)

LS857 cells were cultured in the presence or absence of pioglitazone. Following induction of morphologic differentiation, pioglitazone was withdrawn. After 48 hr of continued culture in the absence of pioglitazone, cells were labeled for 48 hr with BrdUrd, stained, and visualized microscopically. NA, not available.

TLS/CHOP contributes to differentiation arrest and tumorigenesis, however, remains to be elucidated.

The impact of RXR-specific activators on adipocyte differentiation has not been addressed previously. We have demonstrated that RXR-specific retinoids can function as adipogenic regulators through activation of the PPARγ/RXRα heterodimer, and that the adipogenic activity of the heterodimer is maximal when both receptors are bound by their respective ligands. Given that PPARγ is likely to be the biologic receptor mediating the insulin-sensitizing effects of the thiazolidinediones, this observation suggests that RXR-specific ligands may also have insulin-sensitizing activity *in vivo*. Moreover, the insulin-sensitizing effects of thiazolidinedione ligands for PPARγ might be enhanced by simultaneous administration of an RXR-specific ligand.

This study has important implications for the pharmacologic management of liposarcoma in humans. Despite conventional multimodality therapy, 25% to 75% of patients with advanced liposarcoma will die from their disease within 5 years. Our results suggest that the thiazolidinedione class of antidiabetic drugs and RXR-specific retinoids may be useful as a nontoxic alternative to conventional chemotherapy for the treatment of disseminated or locally advanced liposarcoma. Members of the thiazolidinedione class of drugs have undergone extensive preclinical testing as antidiabetic agents. Troglitazone is currently in phase three clinical trials in the United States, and studies have supported its usefulness in noninsulin-dependent diabetes mellitus (31). Although certain thiazolidinediones have been associated with some degree of toxicity in long-term use as insulin sensitizing agents, this should not preclude their use as antineoplastic agents because conventional chemotherapy is associated with far greater toxicity. The ability of thiazolidinediones and RXR-specific retinoids to induce differentiation of liposarcoma cells *in vitro* strongly suggests that these compounds may also be able to stimulate differentiation and growth arrest of human tumors *in vivo*. Finally, although expression of PPARγ is most prominent in fat, it is known to be expressed at some level in select other cell types such as liver and hematopoietic cells (8, 36–38). If PPARγ also plays a role in the regulation of growth and differentiation of these tissues, then this receptor may represent a target for therapeutic intervention in other human malignancies as well.

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1. Mack, T. M. (1995) *Cancer* **75**, 211–244.
2. Chang, H. R., Hajdu, S. I., Collin, C. & Brennan, M. P. (1989) *Cancer* **64**, 1514–1520.
3. Sreekantaiah, C., Ladanyi, M., Rodriguez, E. & Chaganti, R. S. K. (1994) *Am. J. Pathol.* **144**, 1121–1134.
4. Patel, S. R., Burgess, M. A., Plager, C., Papadopoulos, N. E., Linke, K. A. & Benjamin, R. S. (1994) *Cancer* **74**, 1265–1269.
5. Warrell, R. P., Jr., Frankel, S. R., Miller, W. H., Jr., Scheinberg, D. A., Itri, L. M., Hittleman, W. N., Vyas, R., Andreef, M., Tafuri, A., Jakubowski, A., Gabrilove, J., Gordon, M. S. & Dmitrovsky, E. (1991) *N. Engl. J. Med.* **324**, 1385–1393.
6. Warrell, R. P., Jr., de The, H., Wang, Z.-Y. & Degos, L. (1993) *N. Engl. J. Med.* **329**, 177–189.
7. Kliewer, S. A., Umesono, K., Noonan, D., Heyman, R. & Evans, R. M. (1992) *Nature (London)* **358**, 771–774.
8. Tontonoz, P., Hu, E., Graves, R. A., Budavari, A. I. & Spiegelman, B. M. (1994) *Genes Dev.* **8**, 1224–1234.
9. Tontonoz, P., Graves, R. A., Budavari, A. I., Erdjument-Bromage, H., Lui, M., Hu, E., Tempst, P. & Spiegelman, B. M. (1994) *Nucleic Acids Res.* **22**, 5628–5634.
10. Tontonoz, P., Hu, E., Devine, J., Beale, E. G. & Spiegelman, B. M. (1995) *Mol. Cell. Biol.* **15**, 351–357.
11. Sears, I. B., MacGinnitie, M. A., Kovacs, L. G. & Graves, R. A. (1996) *Mol. Cell. Biol.* **16**, 3410–3418.
12. Tontonoz, P., Hu, E. & Spiegelman, B. M. (1994) *Cell* **79**, 1147–1156.
13. Forman, B. M., Tontonoz, P., Chen, J., Brun, R. P., Spiegelman, B. M. & Evans, R. M. (1995) *Cell* **83**, 803–812.
14. Kliewer, S. A., Lenhard, J. M., Wilson, T. M., Patel, I., Morris, D. C. & Lehmann, J. M. (1995) *Cell* **83**, 813–819.
15. Lehmann, J. M., Moore, L. B., Smith-Oliver, T. A., Wilkison, W. O., Wilson, T. M. & Kliewer, S. A. (1995) *J. Biol. Chem.* **270**, 12953–12956.
16. Freytag, S. O., Paielli, D. L. & Gilbert, J. D. (1994) *Genes Dev.* **8**, 1654–1663.
17. Lin, F. & Lane, D. M. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 8757–8761.
18. Wu, Z., Xie, Y., Bucher, N. L. R. & Farmer, S. R. (1995) *Genes Dev.* **9**, 2350–2363.
19. Kamei, Y., Kawada, T., Mizukami, J. & Sugimoto, E. (1994) *Life Sci.* **55**, 307–312.
20. Xue, J. C., Schwarz, E. J., Chawla, A. & Lazar, M. A. (1996) *Mol. Cell. Biol.* **16**, 1567–1575.
21. Levin, A. A., Sturzenbecker, L. J., Kazmer, S., Bosakowski, T., Huselton, C., Allenby, G., Speck, J., Kratzeisen, C., Rosenberger, M. & Lovey, A. (1992) *Ann. N.Y. Acad. Sci.* **669**, 70–86.
22. Heyman, R. A., Mangelsdorf, D. J., Dyck, J. A., Stein, R. B., Eichele, G., Evans, R. M. & Thaller, C. (1992) *Cell* **68**, 397–406.
23. Fletcher, J. A., Kozakewich, H. P., Hoffer, F. A., Lage, J. M., Weidner, N., Tepper, R., Pinkus, G. S., Morton, C. C. & Corson, J. M. (1991) *N. Engl. J. Med.* **324**, 436–443.
24. Seabright, M. A. (1971) *Lancet* **ii**, 971–972.
25. Fletcher, J. A., Gibas, Z., Donovan, K., Perez-Atayde, A., Genest, D. & Morton, C. C. (1991) *Am. J. Pathol.* **138**, 515–520.
26. Green, H. & Kehinde, O. (1974) *Cell* **1**, 113–116.
27. Braissant, O., Foufelle, F., Scotto, C., Dauca, M. & Wahli, W. (1996) *Endocrinology* **137**, 354–366.
28. Fletcher, C. D. M., Akerman, M., Cin, P. D., de Wever, I., Mandahl, N., Mertens, F., Mitelman, F., Rosai, J., Rydholm, A., Sciot, R., Tallini, G., van den Berghe, H., van de Ven, W., Vanni, R. & Willen, H. (1996) *Am. J. Pathol.* **148**, 623–630.
29. Fletcher, J. A., Kozakewich, H. P., Hoffer, F. A., Lage, J. M., Weidner, N., Tepper, R., Pinkus, G. S., Morton, C. C. & Corson, J. M. (1991) *N. Engl. J. Med.* **324**, 436–442.
30. Knight, J. C., Renwick, P. J., Cin, P. D., Van den Berghe, H. & Fletcher, C. D. (1995) *Cancer Res.* **55**, 24–27.
31. Cornelius, P., MacDougald, O. A. & Lane, M. D. (1994) *Annu. Rev. Nutr.* **14**, 99–129.
32. Tontonoz, P., Hu, E. & Spiegelman, B. M. (1995) *Curr. Opin. Genet. Dev.* **5**, 571–576.
33. Yeh, W.-C., Cao, Z., Classon, M. & McKnight, S. L. (1995) *Genes Dev.* **9**, 168–181.
34. Crozat, A., Aman, P., Mandahl, N. & Ron, D. (1993) *Nature (London)* **363**, 640–644.
35. Nolan, J. J., Ludvik, B., Beardson, P., Joyce, M. & Olefsky, J. (1994) *N. Engl. J. Med.* **331**, 1188–1193.
36. Kliewer, S. A., Forman, B. M., Blumberg, B., Ong, E. S., Borgmeyer, U., Mangelsdorf, D. J., Umesono, K. & Evans, R. M. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 7355–7359.
37. Greene, M. E., Blumberg, B., McBride, O. W., Yi, H. F., Kronquist, K., Kwan, K., Hsieh, L., Greene, G. & Nimer, S. D. (1995) *Gene Exp.* **4**, 281–299.
38. Zhu, Y., Alvares, K., Huang, Q., Rao, M. S. & Reddy, J. K. (1993) *J. Biol. Chem.* **268**, 26817–26820.

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Gene Therapy and Translational Cancer Research[1]

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The Pathology B and Experimental Therapeutics-2 study sections sponsored a workshop devoted to recent advances in gene therapy and translational research relevant to cancer. The workshop was highlighted by a historical perspective of gene therapy by Dr. Theodore Friedman (University of California, San Diego, CA). The opening remarks that human gene therapy is not a current success set the tone for a rigorous examination of this approach to cancer treatment. There are currently approximately 300 approved gene therapy trials, 188 of which involve cancer biology or therapy. Gene therapy is defined as the ability to treat disease at the level of the underlying gene defect, whether it is applied to cure genetic diseases, target somatic cells with single gene defect, or treat complex diseases or nongenetic diseases such as AIDS. The obstacles surrounding effective human gene therapy have been studied by the Orkin-Motulsky Committee commissioned by Dr. Harold Varmus, director of the NIH (Bethesda, MD). This committee found human gene therapy to be an immature science with limited understanding of gene regulation and disease models for preclinical studies. Recommendations are to set high standards for clinical studies, encourage and support interdisciplinary studies, and focus on basic issues of molecular virology. This workshop reviewed some recent advances in gene delivery, gene expression, immune manipulation, and the development of molecular targets and stressed that all of these fields will need further advancement to make gene therapy a reality.

As an example of the promise and pitfalls of gene therapy, the pioneering work of Anderson and Blaese was reviewed by Dr. Theodore Friedman, and recent studies were reviewed by Dr. Donald Kohn (Children's Hospital Los Angeles, University of Southern California School of Medicine, Los Angeles, CA). Although the transduction of the ADA[3] gene into the lymphocytes of patients afflicted by immunodeficiency by Anderson and Blaese was accomplished, the data indicate that no therapeutic effect has been gained from this initial study. In a clinical trial started in 1993, three infants with ADA-deficient SCID were given autologous umbilical cord blood $CD34^+$ (CD34 is a marker associated with hematopoietic stem) cells transduced with normal human ADA cDNA. The patients were also treated weekly with i.m. PEG-ADA. Four years later, they continue to produce leukocytes containing ADA cDNA, with approximately 100-fold higher frequencies of gene-containing T lymphocytes (1–10%) than other lineages (0.01–0.1%). In the summer of 1997, the investigators stopped the PEG-ADA replacement for one patient; multiple laboratory and clinical parameters of immune function declined during the 2-month period, and PEG-ADA treatment was reinstituted.

This scientific "proof of principle" but clinical defeat clearly showed that more work was necessary on vector engineering for greater transduction efficiency and gene expression. As such, work to improve vectors for gene delivery has recently offered some promises. These include the improvement of retroviral vectors through pseudotyping. That is, by engineering the VSV-G glycoprotein on the surface of retroviral particles, retroviruses are able to effectively enter the lipid membrane of cells through an unknown mechanism. Other recent advances in the development of improved viral vectors were discussed by Dr. Inder Verma (The Salk Institute, La Jolla, CA), who reviewed some of the evolution of gene therapy from the perspective of the effort to correct the coagulation factor IX deficiency as a gene therapeutic model. Initial studies with retroviral vectors expressing factor IX in fibroblasts resulted in only short-term expression. Expression in myoblasts was achieved through the use of muscle-specific creatine kinase enhancer promoter sequences, with high levels of factor IX production lasting for up to 2 years when transduced myoblasts were implanted into nude mice. However, when the same approach was taken with factor IX-deficient dogs, the dog myoblasts transduced with factor IX-expressing adenoviruses ceased to produce factor IX after 20 days *in vivo*. It became readily apparent that the immunocompetent animals mounted a brisk antibody and cellular immune response to the viruses and transduced cells, thereby eliminating the expression of factor IX. Other models using adenoviral vectors have succumbed to similar limitations by the host immune response to adenoviruses. Thus, new vectors are needed to circumvent these limitations.

An ideal vector is expected to: (*a*) be available at high titers; (*b*) be convenient; (*c*) be reproducible; and (*d*) confer no immune responses. The lentiviruses, such as HIV, have been exploited to provide these characteristics. In particular, Dr. Verma described a vector producing viral particles in which the surface gp120 of HIV has been replaced by the VSV-G protein that allows entry into cells. The HIV-based vectors are able to transduce genes into cells independent of their growth status. Recent studies have demonstrated that the HIV-based vectors are capable of conferring the ectopic expression of genes in neuronal cells of the central nervous system, and there has been

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[3] The abbreviations used are: ADA, adenosine deaminase; PEG, polyethylene glycol; PBCP, peripheral blood progenitor cell; MoMuLV, Moloney murine leukemia virus; LTR, long terminal repeat; GM-CSF, granulocyte macrophage colony-stimulating factor; TIL, tumor-infiltrating lymphocyte; VSV, vesicular stomatitis virus; SCID, severe combined immunodeficiency disease; ES, embryonic stem; AFP, α-fetoprotein.

no evidence of brisk immune responses to the HIV-based vectors.

Some of the problems of high efficiency transfection might become less important if the introduced gene were in a renewable stem cell, or if its expression conferred a survival benefit. Dr. Kenneth Cowan (Medicine Branch, National Cancer Institute, Bethesda, MD) discussed the hematopoietic reconstitution of CD34-selected cells transduced with the multidrug resistant MDR1 gene in patients with metastatic breast cancer as a model of hematopoietic stem cell marking and drug tolerance. His group has conducted two clinical trials of retroviral-mediated transfer of the multidrug resistance gene MDR1 into PBPCs in patients with metastatic breast cancer to determine whether chemotherapy after reinfusion of transduced cells can selectively expand the population of MDR1-marked hematopoietic cells. In the first trial, patients were treated with high-dose ifosfamide, carboplatin, and epirubicin chemotherapy along with hematopoietic stem cell support. In this trial, only one-third of the harvested $CD34^+$ cells were transduced with a retroviral vector expressing the MDR1 gene. After reconstitution, all patients were treated with five cycles of paclitaxel, and the level of MDR1 gene marking in the peripheral blood was analyzed after each cycle of therapy. Three of the four patients had detectable MDR1 gene marking at reconstitution, and two patients had MDR1 marking of granulocytes throughout the five cycles of therapy.

The Cowan group then initiated a second trial in which patients received only $CD34^+$ cells exposed to retroviral gene transduction conditions to avoid the potential competition for engraftment with nontransduced cells. Six patients received genetically altered $CD34^+$ PBPCs; half of the cells were incubated in supernatant from a retroviral producer clone containing the MDR1 gene, and the other half were incubated in supernatant from a retroviral producer clone containing the neomycin resistance (NeoR) gene. A maximum of 1.5×10^6 $CD34^+$-selected cells/kg were transduced with each vector and transfused. In two of six patients, both peripheral granulocyte and monocyte fractions showed no detectable marking early in the course of treatment after reconstitution but later demonstrated evidence of *in vivo* selection of cells containing MDR1; there was no selection of cells containing NeoR. In the remaining four patients, early marking was apparent, but long-term expansion with chemotherapy was not observed. The difference in long-term granulocyte and monocyte marking by MDR1 compared to NeoR in two of six patients suggests MDR1-transduced stem cells were selected *in vivo* by chemotherapy in these patients. This study also demonstrated the safety of using only PBPCs incubated in retroviral supernatant for hematopoietic reconstitution because all six gene therapy patients recovered from myelosuppressive therapy, and each went on to receive eight cycles of intensive chemotherapy.

A similar strategy to genetically alter drug metabolism in hematopoietic cells was taken by Dr. James Doroshow (City of Hope National Medical Center, Duarte, CA). Chemoprotective strategies involve the use of bicistronic vectors capable of expressing genes encoding glutathione peroxidase or glutathione *S*-transferase π. Ectopic expression of glutathione peroxidase confers tolerance to high levels of colchicine and protects cells against oxidative stress, for example, with glucose oxidase exposure. Transduction of the MDR1 gene was also undertaken, and selection for the MDR1 gene was enhanced by the exposure of cells to colchicine. In fact, colchicine was more effective in the selection of MDR1-expressing cells than G418 selection. When applied to mouse and human $CD34^+$ cells, however, the transduction efficiency of MDR1 was very low (about 20%). Alternative gene transfer strategies such as lipofection appear to be feasible in human $CD34^+$ cells, resulting in up to 50% marked cells. Thus, the limitations of efficient gene transfer require additional studies of alternative delivery options, such as lipofection, or the development of better vectors.

Even with the efficient delivery of genes and a way to enrich for cells expressing those gene products, pitfalls remain in stable gene expression. Dr. Donald Kohn discussed retroviral vectors with multiple modifications in the *cis*-elements implicated in transcriptional silencing. These modified vectors showed a >1000-fold higher expression of NeoR or chloramphenicol acetyltransferase reporter gene than did standard MoMuLV LTR vectors in murine ES cells. The modified MND LTR directed expression of the E-GFP reporter in essentially 100% of transduced ES cells, whereas unmodified MoMuLV LTR was active in only 1–5% of transduced ES cells. The modified vectors have now been evaluated in the serial murine bone marrow transplantation model and were found to be significantly less methylated than MoMuLV LTR. The modified vectors were expressed in >90% of hematopoietic colony-forming units, whereas MoMuLV LTR was expressed in only 10–20% of hematopoietic colony-forming units. The MND vector also showed a higher level of expression in T and B lymphocytes produced in the mice transplanted with transduced bone marrow. Lentiviral vectors are currently being examined to see if they will increase the transduction of primitive pluripotent human hematopoietic stem cells.

In the long term, the application of gene therapy methods is limited by our knowledge of how genes are silenced or regulated within a chromosomal environment. Dr. Robert Oshima (The Burnham Institute, La Jolla, CA) presented recent findings regarding the transcriptional regulation of transgenes and sequences that regulate chromatin structure and gene expression. Many or most genes integrated into cells and animals are subject to position effects that alter the expression of the gene. The Oshima laboratory has discovered *cis*-acting sequences flanking the keratin 18 gene (K18) that confer integration site-independent and copy number-dependent expression of both the K18 gene and two tested heterologous genes in transgenic mice. They have found that the smallest K18-flanking fragment able to confer position-independent expression on the metallothionein-human growth hormone transgene is a 340-bp fragment composed primarily of an Alu-repetitive element. This element, which is transcribed by RNA polymerase III, was found to protect the transgene in an orientation-dependent manner, with the most effective arrangement consisting of inverted repeats flanking the transgene. Furthermore, when interposed between a synthetic telomere that nucleates repressive chromatin structure and a URA3 reporter gene, the Alu sequence was able to block chromatin-mediated repression in yeast. In a genetic background that relieved chromatin-mediated repression due to the absence of the Sir3 gene product, the Alu sequence had no effect. This confirmed that the activity of the Alu was due to blocking chromatin-mediated repression and not simply to elevating the transcriptional activity of the URA3 gene. This effect, like the protection afforded in transgenic mice, was

dependent on the orientation of the Alu sequence. These results indicate that much of the locus control region, like the activity of the K18-flanking sequences, is associated with a small 340-bp Alu sequence, and that the same element was active in yeast to block chromatin-mediated repression. *cis*-acting elements like the K18 Alu fragment may facilitate the expression of other genes when integrated into random locations within the genome. It also suggests that a subset of Alu sequences may function to define regulatory domains within the human genome and leads to speculation about the function of Alu elements in gene regulation and during evolution.

Just as knowledge of the basic biology surrounding viral transfection and gene expression will advance translational achievements in gene therapy, a better knowledge of the immune system is furthering its potential application toward cancer vaccine therapy. Dr. James Economou (University of California Los Angeles School of Medicine, Los Angeles, CA) discussed the use of genetically engineered dendritic cell animal models. Dendritic cells are professional antigen-presenting cells that process antigens and present them to T cells as part of a complex reaction leading either to tolerance or to CTL-mediated elimination of noncognate cellular targets. As a model, the melanoma MART-1 peptide antigen was used to engineer dendritic cells that express MART-1. CTLs readily recognize dendritic cell clones transduced with MART-1 expression vector. The tumor model is a transfected MART-1-expressing murine fibrosarcoma that is tumorigenic but is normally poorly immunogenic. Murine bone marrow cells were treated with GM-CSF and interleukin 4 before being harvested for dendritic cells, which were transduced with the MART-1 adenoviral expression vector and injected into tumor-bearing animals. The dendritic cell-treated animals demonstrated a marked suppression of tumor growth as compared to control-treated animals. In 20% of animals, no tumor growth was detected, whereas the remaining 80% demonstrated a delayed outgrowth after treatment with engineered dendritic cells. Curiously, repeated immunizations worsened the outcome that correlated with a decrease in the CTL assay. In addition, both CD4 and CD8 cells were necessary for protection against tumor formation. Clinical trials are ongoing with adenovirus MART-1-transduced cells. As additional proof of principle, the α-fetoprotein was exploited in the immune-mediated killing of hepatocellular carcinoma cells. With the BWIC3 hepatoma cell line as a tumor model, immunization with adenovirally transduced AFP dendritic cells resulted in a marked protection against tumor formation. Additional studies revealed two dominant AFP peptides presented by HLA-2 molecules that play a role in CTL response. Through specific peptide loading experiments, it was demonstrated that specific lysis of AFP-expressing cells can be elicited. These studies suggest that engineered dendritic cells and specific immunogenic peptide loading may provide additional means to attack cancer cells.

Dr. Edmund C. Lattime (Thomas Jefferson University, Philadelphia, PA) discussed the use of vaccinia virus for immunotherapy. The principal goal of immunotherapeutic strategies in tumors is the induction of a systemic cell-mediated antitumor response that would eliminate both primary and metastatic tumor foci. Applying earlier findings by Pardoll and coworkers, the Lattime group has proposed an approach to immunological gene therapy in which the genes for immune-enhancing cytokines and cell surface antigens can be transfected into tumor cells *in situ* using vaccinia virus vectors. Lattime has evaluated recombinant vaccinia encoding GM-CSF as a candidate molecule to enhance the generation of tumor-specific immunity by enhancing antigen presentation. In murine studies, they show that the GM-CSF gene is expressed both early and late in the course of therapy, and that preexisting or developing immunity to vaccinia does not prevent GM-CSF gene expression. In a Phase I clinical study of intralesional recombinant vaccinia-GM-CSF in s.c. melanoma metastases, they found that the recombinant can be given safely, and the GM-CSF gene is expressed both early and late in the treatment course. Intralesional injection of the recombinant results in the regression of injected lesions, which is most probably due to the antivaccinia inflammatory response. In addition, in a number of patients, they have found that distant uninjected lesions regress, suggesting the development of systemic antitumor immunity. These studies support the use of vaccinia recombinants for *in situ* use as vectors for inserting immune active molecules into tumor cells.

Dr. Alfred E. Chang (University of Michigan, Ann Arbor, MI) discussed the applications of gene transfer in the adoptive immunotherapy of cancer. Adoptive immunotherapy of cancer involves the passive transfer of immune cells that are capable of conferring systemic immunity to the host to mediate tumor regression. Traditionally, this has involved the isolation and expansion of T cells from either TILs or tumor-draining lymph nodes. With the advent of gene transfer methodologies, new techniques to enhance the generation of immune T cells have been explored. TILs represent lymphoid cells derived from tumors that are disaggregated *ex vivo* and cultured in interleukin 2. A significant impediment to generating therapeutic TIL resides in the inherent immunogenicity of the tumor from which the TILs are derived. In animal models using poorly immunogenic tumors, the therapeutic efficacy of the TILs is limited. A novel variation to altered TIL reactivity has been developed at the University of Michigan. In animal studies, tumors treated by the *in vivo* transfer of an allogeneic MHC class I gene complexed with liposomes resulted in the expression of the class I molecules by tumor cells. Importantly, this also resulted in tumor regression and the induction of T cells that were not only reactive to transfected tumor cells, but also to unmodified cancer cells. They have previously reported evidence of gene expression in advanced melanoma patients treated by *in vivo* inoculation of the tumor with DNA/liposome complexes containing a foreign MHC class I gene. Based on these initial observations, they are currently evaluating the immune reactivity of TILs derived from tumors modified by direct *in vivo* gene transfer using an allogeneic class I MHC gene, HLA-B7, in patients with stage IV melanoma. In *in vitro* assays, they have confirmed an enhanced reactivity of TILs derived from patients inoculated with the foreign class I gene. Another method of T-cell therapy has been to activate tumor-draining lymph node cells, which they have demonstrated to have potent antitumor reactivity on adoptive transfer in preclinical studies. In a poorly immunogenic tumor model, they have found that genetically modifying tumor cells to secrete GM-CSF results in significant tumor reactivity of the tumor-draining lymph node cells. They hypothesize that the local secretion of GM-CSF promotes the recruitment and expansion of antigen-presenting dendritic cells. This approach is being evaluated in a clinical trial at the University of Michigan in which patients with stage IV melanoma are vaccinated with autologous melanoma tumor cells transduced to secrete GM-CSF. Thus far, the vaccine sites have demonstrated a local influx of

dendritic cells. They have been able to define autologous tumor cell reactivity of the vaccine-primed lymph node cells. One patient with metastatic disease treated with these adoptively transferred cells has experienced a complete response. They feel that the use of gene transfer techniques may result in more effective methods to generate tumor-specific T cells for cellular therapy.

Even in the perfect scenario, in which all of the problems with gene delivery and expression are solved, these technologies may be fruitless without a good understanding of the basic defects in cancer biology and the correct choices of molecular targets for gene therapy. A part of this workshop revolved around the more basic issues of the use of alternative cancer gene therapy approaches and translational research applied to molecular profiling and anticancer drug discovery. Dr. Ming-Chie Hung (The University of Texas M. D. Anderson Cancer, Houston, TX) took a novel approach toward cancer gene therapy by exploiting the tumor suppression activity of E1A in HER-2/neu-overexpressing cancer cells. The HER-2/neu (also named c-*erb*B-2) oncogene is known to be overexpressed in many human cancers, including breast, ovarian, lung, gastric, and oral cancers. In animal models, HER-2/neu overexpression was shown to enhance malignancy and metastasis phenotypes. Repression of HER-2/neu overexpression suppresses the malignant phenotypes of HER-2/neu-overexpressing cancer cells, suggesting that HER-2/neu may serve as an excellent target for developing anticancer agents. The Hung group has previously shown that adenovirus-5 E1A gene products inhibit overexpression of the HER-2/neu oncogene and accordingly suppress transformation induced by HER-2/neu. Their results indicate that cationic liposomes or an adenoviral vector can efficiently deliver E1A into tumor cells in mice, and this results in the suppression of tumor growth and longer survival of the mice. Based on these results, a Phase I gene therapy clinical trial entitled "Phase I Study of E1A Gene Therapy for Patients with Metastatic Breast or Ovarian Cancer that Overexpresses HER-2/neu" was initiated at M. D. Anderson Cancer Center (P. I. Gabriel Hortobagyi). The data from the clinical trial indicate that the E1A gene can be expressed in treated patients, and HER-2/neu down-regulation in tumor cells is readily detectable. A maximum tolerable dose is also identified. The result suggests that E1A-liposome gene therapy is feasible, and additional clinical trials on patients with less-malignant cancer are required to evaluate therapeutic efficacy.

Dr. Patricia Steeg (National Cancer Institute) discussed Nm23 as a potential therapeutic target. Nm23 expression is reduced in metastatic cells, and the introduction of Nm23 into certain cancer cell lines has reduced their metastatic potential. One approach to the problem of metastasis is to up-regulate the expression of Nm23. As such, Steeg and coworkers studied the Nm23 promoter and found three breast-specific elements consisting of a consensus 5′-ACAAAG-3′ site and NF1 and ETS binding sites. Although the regulation of Nm23 expression has not been exploited for therapeutic purposes, the Steeg group has used the COMPARE computer algorithm and Nm23 expression as a marker of tumor metastatic potential to examine the *in vitro* antiproliferative activity of chemotherapeutic drugs on human breast carcinoma and melanoma cell lines. None of 171 compounds in clinical use or under development and only 40 of 30,000 repository compounds exhibited preferential growth inhibition of low-Nm23-expressing metastatically aggressive cell lines with a Pearson correlation coefficient of ≤ -0.64. Characterization of one compound, NSC 645306, is presented including *in vivo* activity in a hollow fiber assay. The data demonstrate a novel approach using molecular markers and the NCI 60 human cancer cell line panel to identify potential drugs for aggressive human tumors.

Dr. Chi Van Dang (Johns Hopkins University School of Medicine, Baltimore, MD) presented recent studies on c-Myc target genes and their therapeutic implications. Based on the frequency of genetic alterations of c-*myc* in human cancers, it can be estimated that approximately 70,000 United States cancer deaths per year are associated with changes in the c-*myc* gene or its expression. Given that c-*myc* may contribute to one-seventh of United States cancer deaths, the understanding of c-*myc* is important to our intellectual armamentarium against cancer. The c-Myc gene encodes an oncogenic helix-loop-helix-leucine zipper transcription factor that acts as a heterodimer with its partner protein, Max, to activate genes regulating cell growth, differentiation, and cell death. To further understand c-Myc function, the Dang group has identified a set of putative c-Myc-responsive genes through the application of cDNA representational difference analysis. They identified 17 up-regulated genes and 3 down-regulated genes in a rat fibroblast system that is susceptible to c-Myc-mediated transformation. Two novel genes, *rcl* and *JP1*, cause anchorage-independent growth, although at a very reduced efficiency compared to c-Myc. Three other genes, *KAP1*, *Mer5*, and *LDH-A* (encoding lactate dehydrogenase A) are nontransforming; however, LDH-A is required for the establishment of three-dimensional spheroid growth in soft agar. Furthermore, *LDH-A* participates in c-Myc-mediated glucose deprivation-induced apoptosis. This observation is being exploited for therapeutic purposes. However, in addition to the study of target genes, Dang and coworkers observed that overexpression of c-*myc* causes Colcemid-treated human and rodent cells to replicate DNA without chromosomal segregation and become either apoptotic or polyploid. c-Myc protein levels were further correlated with the sensitivity of 60 human cancer cell lines to tubulin binding drugs, suggesting that this novel phenotype of c-Myc sensitizes cells to this class of antitumor drug. This approach toward studying the genetic program enforced by c-Myc in cellular transformation has led to the identification of novel connections between cellular metabolism and transformation as well as the identification of novel genes that probably account for some of the transforming activities of c-Myc.

The studies presented at this workshop provide a glimpse of the past and present and a projection of the future of cancer gene therapeutics and novel therapeutic approaches based on sound molecular underpinnings. Although significant progress has been achieved in our understanding of the limitations of gene therapy by suboptimal vectors, host immunological responses to the vectors, and the lack of long term stable expression, the major challenge that limits clinical translation remains in achieving efficient gene delivery to target tissues. As such, gene therapy that exploits the host immune system and the development of novel small molecular therapeutics might hold promise for the future. Whereas setbacks in gene therapy were clearly recognized and discussed, there was a unique level of enthusiasm that many of these obstacles could be overcome with meticulously designed basic and clinical studies.

CHAPTER 5

GENE-BASED THERAPY

Stephen L. Eck and James M. Wilson

Advances in molecular and cellular biology have described the proteins that mediate many disease processes, while DNA technology provides ready access to the genes that control these events. The size, complexity, and cellular inaccessibility of these proteins make their delivery or modification by conventional pharmacological means impossible. Gene therapy overcomes these barriers by the selective introduction of recombinant DNA into tissues so that the biologically active proteins can be synthesized within the cells whose function is to be altered. As such, delivery of recombinant DNA has become a central issue in all gene therapy strategies. A variety of DNA delivery systems have been developed based on viral life cycle pathways, liposome encapsulation, direct injection, and complexation with carrier proteins. Although originally envisioned as a treatment for inherited single-gene defects, gene therapy has found applications in acquired illnesses such as cancer, cardiovascular and infectious diseases. This chapter provides an introduction to the therapeutic issues and current strategies being explored to apply gene therapy to this wide range of diseases.

SCOPE OF GENE THERAPY

Therapeutic gene transfer is not a new concept (Wolff and Lederberg, 1994). More than two decades before the first gene transfer took place in a clinical setting, Edward Tatum speculated: "We can even be somewhat optimistic on the long-range possibility of therapy by the isolation or design, synthesis, and introduction of new genes into defective cells of particular organs" (Tatum, 1966). The treatment of human disease by gene transfer originally was envisioned as a means to treat diseases arising from single-gene defects. Inherited diseases encompass a wide range of disorders wherein a defective gene leads to the failure to synthesize a particular protein or to the synthesis of an abnormal protein. In either event, the absence of the normal protein can lead to a variety of clinical manifestations depending on the structural or enzymatic role that protein normally plays in the cell. Such conditions range from mild disorders that require no treatment (*e.g.*, color blindness) to life-threatening diseases (*e.g.*, hemophilia, cystic fibrosis). These diverse diseases are, in general, inadequately treated by conventional pharmacological means. Therapy based on the replacement f the missing or defective protein (such as factor VIII for hemophilia, transfusions for sickle cell disease, and adenosine deaminase for severe combined immunodeficiency syndrome) is available for only a few of these disorders. Furthermore, these therapies are only partially effective in ameliorating the manifestations of the disease and are accompanied by significant complications. For most genetic diseases, providing the missing protein in a therapeutic fashion is not feasible due to the complex and fragile nature of the protein and the need to deliver the protein to a specific subcellular location (*i.e.*, cell surface expression, lysosomal localization, *etc.*). Transplantation of the major affected organ has been done in some instances (*e.g.*, bone marrow transplantation for sickle cell disease, or liver transplantation for hyperlipidemias), but this also has severe limitations of organ availability and adverse consequences arising from the immune suppression required to prevent rejection of an allogeneic tissue.

Providing a normal copy of the defective gene to affected tissues would circumvent the problem of delivering complex proteins, as the protein could be synthesized within the cells using the normal cellular pathways. Although the defective gene is present in all cells of an individual with an inherited disorder, only a few tissues or organs actually express the gene and therefore are affected. Defects in genes that function in all cells of the body (so-called housekeeping genes) usually result in such severe abnormalities that embryonic development cannot occur. The limited number f tissues affected by most inherited disorders greatly simplifies the requirements for effective gene therapy, since a functional copy f the gene need be

provided only to those tissues that actually require it. The goal of gene therapy, therefore, is t genetically correct th defect in only part f the body. Since this type f therapy is designed not t alter the genetic structure of reproductive organs, it does not prevent the genetic disorder from being passed on to subsequent generations. It is envisioned, however, as a powerful tool to ameliorate or reverse the metabolic consequences in the treated individual. Targeting of the therapeutic gene to a specialized tissue is an area f tremendous interest in all applications of gene therapy. Furthermore, if the gene transfer can be targeted to the major affected organs, then side effects arising from ectopic gene expression in nontarget cells might be avoided. As with other pharmaceutical agents, cell-specific targeting has the advantage of decreasing the effective volume of distribution and the amount of gene transfer agent needed. Such cell-specific delivery systems are not yet available for either drugs or genetic material, but it can be reasonably expected that the explosion of interest in gene therapy will result in new methods that are applicable to the delivery f DNA and conventional pharmaceutics alike. DNA delivery systems are being developed using a variety of chemical, physical, and biologic agents.

The earliest human gene transfer experiments began in 1989 with lymphocyte marking studies. While offering no therapeutic benefit, these initial studies showed that gene transfer could be safely carried out and provided insight into many of the technical difficulties of human gene transfer (Rosenberg *et al.*, 1990). Lymphocytes were likely targets for initial gene therapy attempts because they can be isolated easily and manipulated *ex vivo*. Thus, tissue targeting can be effected by physical removal and manipulation of the recipient cells, rather than by design of the gene delivery system, which has so far proved difficult. Lymphocytes were also attractive because they are the cellular locus of several inherited and acquired disorders (*e.g.*, severe combined immunodeficiency, HIV infection, graft versus host disease, and a variety of malignancies). Furthermore, in addition to being readily isolated, lymphocytes may be expected to be long-lived on return to the recipient and therefore can potentially provide lasting benefits in chronic disorders. Thus, lymphocyte gene transfer provides an important model for gene therapy and continues to be developed for many disorders. In September 1990, the first human gene therapy trial with therapeutic potential began. The *ex vivo* gene transfer of adenosine deaminase (ADA) gene into the lymphocytes of a child with what is normally a lethal deficiency of this enzyme was carried out at the National Institutes of Health (Anderson *et al.*, 1990). The results of this trial, which are yet to be published in detail, were encouraging and have spawned the development of many new gene therapy trials.

The majority of gene therapy trials under way are for the treatment of acquired disorders such as AIDS, malignancies, and cardiovascular disease, rather than diseases arising from single gene defects (Table 5–1). The application of gene therapy to acquired disorders has proceeded faster than applications for single-gene defects for several reasons. Principle among these reasons is that the long-term gene expression (months t years) that is likely necessary to treat genetic diseases has been difficult t achieve. The availability of a large pool of candidate patients with severe and immediately life-threatening acquired disorders (most notably cancer and AIDS) provides a clinical setting to develop new strategies for DNA delivery that may be applied later to inherited disorders. In contrast to the inherited diseases where a genetic defect has been well characterized, in most applications of gene therapy to acquired illnesses, the molecular basis of the disease is less well understood. Rather than correct a known underlying defect, the approach has been to add new molecular functions that are capable of altering the course of the disease, or to block an existing function, rather than correct an underlying deficiency.

General Considerations in Gene Therapy

Inherited Disorders. The insertion of a new gene that ultimately corrects a deficiency requires that the new gene product is present in sufficient amounts to achieve a therapeutic effect. The level of protein function necessary to achieve complementation of the defect varies widely among genetic diseases. Often this can be estimated from clinical observations comparing the severity of the disease with the extent of deficiency. This is seen in the hemophilias, where the extent of bleeding complications is roughly proportional to the extent of the deficiency. Such estimates are not possible in other disorders such as cystic fibrosis, where the amount of cystic fibrosis transport regulator (CFTR) gene expression, in the airway and in other epithelial cells, necessary to achieve therapeutic benefit is not known. Here, the severity of the illness correlates with the type of genetic defect, rather than with the level of protein expression. These issues become more complex in diseases where gene expression must be carried out in a highly regulated fashion. One such example is the thalassemias, which arise from defects in the synthesis of either the α or β chain of hemoglobin. Excessive production of either subunit chain by an unregulated therapeutic gene transfer could be as harmful as the disease itself.

Acquired Disorders. Mechanistically, gene therapy for acquired disorders is potentially more flexible, in terms of the inserted DNA, than gene therapy for inherited disorders. In inherited disorders, a single defective gene that causes the disorder typically is the subject of intervention. By contrast, in acquired diseases, either a defective gene

Table 5–1
Therapeutic Gene Therapy Trials Approved by the Recombinant DNA Advisory Committee f the National Institutes of Health.*

PROTOCOL TITLE	PRINCIPAL INVESTIGATOR	DATE OF APPROVAL
Gene Therapy of Patients with Advanced Cancer Using Tumor Infiltration Lymphocytes Transduced with the Gene Coding for Tumor Necrosis Factor.	S.A. Rosenberg	7/31/90
Immunization of Cancer Patients Using Autologous Cancer Cells Modified by Insertion of the Gene for Tumor Necrosis Factor (TNF)	S.A. Rosenberg	10/7/91
Immunization of Cancer Patients Using Autologous Cancer Cells Modified by Insertion of the Gene for Interleukin-2 (IL-2).	S.A. Rosenberg	10/7/91
Ex vivo Gene Therapy of Familial Hypercholesterolemia.	J.M. Wilson	10/8/91
Treatment of Severe Combined Immune Deficiency (SCID) Due to Adenosine Deaminase (ADA) Deficiency with Autologous Lymphocytes Transduced with the Human ADA Gene: An Experimental Study	R.M. Blaese	2/10/92
Immunotherapy of Malignancy by *In vivo* Gene Transfer into Tumors	G.J. Nabel	2/10/92
Gene Transfer for the Treatment of Cancer.	S.M. Freeman	2/10/92
Gene Therapy for the Treatment of Recurrent Glioblastoma Multiforme with *in vivo* Tumor Transduction with the Herpes Simplex–Thymidine Kinase Gene/Ganciclovir System.	K.W. Culver	3/1/92
A Phase I Study, in Cystic Fibrosis Patients, of the Safety, Toxicity, and Biological Efficacy of a Single Administration of a Replication Deficient, Recombinant Adenovirus Carrying the cDNA of the Normal Human Cystic Fibrosis Transmembrane Conductance Regulator Gene in the Lung.	R.G. Crystal	5/17/92
Phase I Study of Cytokine-Gene Modified Autologous Neuroblastoma Cells for Treatment of Relapsed/ Refractory Neuroblastoma.	M.K. Brenner	6/1/92
Gene Therapy for the Treatment of Brain Tumors Using Intra-Tumoral Transduction with the Thymidine Kinase Gene and Intravenous Ganciclovir	E. Oldfield	6/1/92
Immunization with HLA-A2 Matched Allogeneic Melanoma Cells that Secrete Interleukin-2 in Patients with Metastatic Melanoma.	B. Gansbacher	6/2/92
Immunization with Interleukin-2 Secreting Allogeneic HLA-A2 Matched Renal Cell Carcinoma Cells in Patients with Advanced Renal Cell Carcinoma.	B. Gansbacher	6/2/92
Clinical Protocol for Modification of Oncogene and Tumor Suppressor Gene Expression in Non-Small Cell Lung Cancer (NSCLC).	J.A. Roth	9/15/92
Gene Therapy of Cancer: A Pilot Study of IL-4 Gene Modified Antitumor Vaccines.	M.T. Lotze	9/15/92
Gene Therapy of Cystic Fibrosis Lung Diseases Using E1 Deleted Adenoviruses: A Phase I Trial.	J.M. Wilson	12/3/92
Cystic Fibrosis Gene Therapy Using an Adenovirus Vector: *In vivo* Safety and Efficacy in Nasal Epithelium.	M.J. Welsh	12/4/92
Phase I Study of Non-Replicating Autologous Tumor Cell Injections Using Cells Prepared With or Without Granulocyte-Macrophage Colony Stimulating Factor Gene Transduction in Patients with Metastatic Renal Cell Carcinoma.	J. Simons	3/1/93
Administration of Neomycin Resistance Gene Marked EBV Specific Cytotoxic T Lymphocytes to Recipients of Mismatched-Related or Phenotypically Similar Unrelated Donor Marrow Grafts.	H.E. Heslop	3/2/93
A Phase I Study of Gene Therapy of Cystic Fibrosis Utilizing a Replication Deficient Recombinant Adenovirus Vector to Deliver the Human Cystic Fibrosis Transmembrane Conductance Regulator cDNA to the Airways.	R.W. Wilmott	3/2/93
Gene Therapy for Cystic Fibrosis Using E1 Deleted Adenovirus: A Phase I Trial in the Nasal Cavity.	R.C. Boucher	3/2/93
A Phase I Trial of Human Gamma Interferon-Transduced Autologous Tumor Cells in Patients With Disseminated Malignant Melanoma.	H.F. Seigler	6/7/93
Use of Safety-Modified Retroviruses to Introduce Chemotherapy Resistance Sequences into Normal Hematopoietic Cells for Chemoprotection During the Therapy of Ovarian Cancer: A Pilot Trial.	A.B. Deisseroth	6/7/93
Immunotherapy for Cancer by Direct Gene Transfer into Tumors	G.J. Nabel	6/7/93
Gene Therapy for Gaucher Disease: *Ex vivo* Gene Transfer and Autologous Transplantation of CD34+ Cells.	J.A. Barranger	6/7/93
Retroviral Mediated Transfer of the cDNA for Human Glucocerebrosidase into Hematopoietic Stem Cells of Patients with Gaucher Disease.	S. Karlsson	6/7/93
A Preliminary Study to Evaluate the Safety and Biologic Effects of Murine Retroviral Vector Encoding HIV-1 Genes [HIV-IT(V)] in Asymptomatic Subjects Infected with HIV-1.	J.E. Galpin	6/7/93

*The protocols listed were approved through August, 1994. Detailed protocols for these clinical trials are published in the monthly journal *Human Gene Therapy*.

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rapeutic Gene Therapy Trials Approved by the Recombinant DNA Advisory Committee f the itional Institutes of Health.* (*Continued*)

TOCOL TITLE	PRINCIPAL INVESTIGATOR	DATE OF APPROVAL
Molecular Genetic Intervention for AIDS—Effects of a Transdominant Negative Form of Rev.	G.J. Nabel	6/7/93
e Therapy for the Treatment of Recurrent Pediatric Malignant Astrocytomas with *in vivo* Tumor nsduction with the Herpes Simplex–Thymidine Kinase Gene.	C. Raffel	6/8/93
man MDR Gene Transfer in Patients with Advanced Cancer.	C. Hesdorffer	6/8/93
e Therapy for Human Brain Tumors Using Episome-Based Antisense cDNA Transcription of Insulin-Like owth Factor I.	J. Han	6/8/93
munization of Malignant Melanoma Patients with Interleukin 2–Secreting Melanoma Cells Expressing efined Allogeneic Histocompatibility Antigens.	T.K. Das Gupta	9/10/93
roviral Mediated Transfer of the Human Multi-Drug Resistance Gene (MDR-1) into Hematopoietic Stem ls During Autologous Transplantation after Intensive Chemotherapy for Breast Cancer.	J. O'Shaughnessy	9/9/93
ne Therapy for Recurrent Pediatric Brain Tumors.	L.E. Kun	9/9/93
Phase I Clinical Trial to Evaluate the Safety and Effects in HIV-1 Infected Humans of Autologous mphocytes Transduced with a Ribozyme that Cleaves HIV-1 RNA.	F. Wong-Staal	9/10/93
ctically Engineered Autologous Tumor Vaccines Producing Interleukin-2 for the Treatment of letastatic Melanoma.	J.S. Economou	9/10/93
thecal Gene Therapy for the Treatment of Leptomeningeal Carcinomatosis.	E.H. Oldfield	12/2/93
ction of Colon Carcinoma Patients with Autologous Irradiated Tumor Cells and Fibroblasts netically Modified to Secrete Interleukin-2.	R.E. Sobol	12/2/93
trovirus-Mediated Transfer of the cDNA for Human Glucocerebrosidase into Peripheral Blood npopulating Cells of Patients with Gaucher's Disease.	F. Schuening	12/2/93
Open Label, Phase I/II Clinical Trial to Evaluate the Safety and Biological Activity of HIV-IT (V) HIV-1 IIIBenv/Retroviral Vector) in HIV-1 Infected Subjects.	R. Haubrich	12/3/93
Phase I Trial of B7-Transfected Lethally Irradiated Allogeneic Melanoma Cell Lines to Induce Cell diated Immunity Against Tumor-Associated Antigens Presented by HLA-A1 in Patients with Stage IV lanoma.	M. Sznol	12/3/93
ase I Study of Immunotherapy of Advanced Colorectal Carcinoma by Direct Gene Transfer into Hepatic letastases.	J. Rubin	12/3/93
ptive Immunotherapy of Melanoma with Activated Lymph Node Cells Primed *in vivo* with Autologous mor Cells Transduced with the IL-4 Gene.	A.E. Chang	12/3/93
ene Therapy for Cystic Fibrosis Using Cationic Liposome Mediated Gene Transfer: A Phase I Trial of Safety and Efficacy in the Nasal Airway.	E.J. Sorscher	12/3/93
enovirus-Mediated Gene Transfer of CFTR to the Nasal Epithelium and Maxillary Sinus of Patients with ystic Fibrosis.	M.J. Welsh	12/3/93
Phase I Study of Immunization with Gamma Interferon Transduced Neuroblastoma Cells.	J. Rosenblatt	3/3/94
Phase I/II Pilot Study of the Safety of the Adoptive Transfer of Syngeneic Gene-Modified Cytotoxic Lymphocytes in HIV-Infected Identical Twins.	R. Walker	3/3/94
xpression of an Exogenously Administered Human Alpha-1-Antitrypsin Gene in the Respiratory Tract of Humans.	K. Brigham	3/3/94
ase I Study of Immunotherapy for Metastatic Renal Cell Carcinoma by Direct Gene Transfer into letastatic Lesions.	N. Vogelzang	3/4/94
Phase I Study of Immunotherapy of Malignant Melanoma by Direct Gene Transfer.	E. Hersh	3/4/94
Phase I Trial of a Polynucleotide Augmented Anti-Tumor Immunization of Human Carcinoembryonic Antigen in Patients with Metastatic Colorectal Cancer.	D. Curiel	6/9/94
linical Trial to Assess the Safety, Feasibility, and Efficacy of Transferring a Potentially Anti-arthritic Cytokine Gene to Human Joints with Rheumatoid Arthritis.	C.H. Evans	6/9/94
se of Safety-Modified Retroviruses to Introduce Chemotherapy Resistance Sequences into Normal Hematopoietic Cells for Chemoprotection During the Therapy of Breast Cancer: A Pilot Trial.	A. Deisseroth	6/9/94

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OCOL TITLE	PRINCIPAL INVESTIGATOR	DATE OF APPROVAL
troviral Mediated Gene Transfer of the Fanconi Anemia Complementation Group C Gene to lematopoietic Progenitors of Group C Patients.	J.M. Liu	6/9/94
cal Protocol for Modification of Tumor Suppressor Gene Expression and Induction of Apoptosis in Non-ll Cell Lung Cancer (NSCLC) with an Adenovirus Vector Expressing Wildtype p53 and Cisplatin.	J.A. Roth	6/10/94
ection of Glioblastoma Patients with Tumor Cells Genetically Modified to Secrete Interleukin-2 (IL-2): phase I Study.	R.E. Sobol	6/10/94
Gene Therapy Using Direct Injection of Tumor with Genetically Engineered Autologous Fibroblasts.	M.T. Lotze	6/10/94
e I/II Study of Autologous Human GM-CSF Gene Transduced Prostate Cancer Vaccines in Patients with tastatic Prostate Carcinoma.	J. Simons	8/3/94

protocols listed were approved through August, 1994. Detailed protocols for these clinical trials are published in the monthly journal *Human Gene Therapy.*

hat directly contributes to the disorder, or a gene that mediates an unrelated biochemical process, may be the basis for interventi n. This diversity of approaches in treating acquired illnesses is illustrated in the gene therapy strategies that have been proposed for treating AIDS and various cancers. Treatment of HIV infection potentially could rely on the interruption of viral processes that directly contribute to the pathogenesis of AIDS. This could be achieved by several means, including inserting a gene that produces antisense mRNA, catalytic RNA (ribozymes), or a dominant negative mutant protein.

Vaccination. Gene transfer-mediated vaccination has become a rapidly expanding field and is applicable to the treatment of both noninfectious and infectious diseases.

Vaccination Against Noninfectious Diseases. Gene therapy for neoplastic diseases includes efforts to engineer an immune response to tumor cells. The idea that tumor cells can be used to elicit an antitumor immune response is founded in rare clinical observations of spontaneous tumor regression, the fact that some tumors are more common in immunocompromised hosts, and the discovery of tumor-associated antigens on many different tumor types. The general strategies proposed include transducing autologous tumor cells (or tumor infiltrating lymphocytes) to secrete a specific cytokine (*e.g.*, tumor necrosis factor, interleukin-2, interleukin-4, interferon gamma, *etc.*), inducing tumor cell expression of a strong rejection antigen (*e.g.*, allogeneic major histocompatibility or MHC molecules), and inducing tumor cell expression of lymphocyte costimulatory molecules (*e.g.*, B7-1). Several of these approaches have reached the stage f clinical trials, but the data from these phase I studies are limited and insufficient to indicate their therapeutic effectiveness (for reviews of this topic, *see* Nabel *et al.*, 1994).

Vaccination Against Infectious Diseases. The use of gene transfer to stimulate immunity to infectious agents also is under investigation. Insertion of DNA sequences that encode key antigens from pathogenic agents (subunit vaccines) would allow for the cellular synthesis and presentation of these antigens in a manner that physiologically mimics their presentation during infections, without the risks of actual exposure to the pathogenic organism. This could have significant implications in the development of an HIV vaccine where the safety implications of a live, attenuated HIV vaccine are awesome.

Obstacles to Gene Therapy

The therapeutic applications of gene transfer technology increase with each discovery of a new cellular process. At present, our ability to develop clinically efficacious therapies from scientifically sound principles is limited by several problems that, to some extent, plague all gene therapy strategies. For the foreseeable future, gene therapy is limited to somatic cells (nongerm-line cells). How these cells in a given tissue are targeted by the DNA delivery method has been an area of intense interest. Once the gene has been successfully transferred, the duration of transgene expression becomes important. Finally, the DNA vector itself must be analyzed for its potential to cause unwanted side effects (Jolly, 1994).

DNA Delivery and Pharmacokinetics. The delivery of exogenous DNA and its processing by target cells require the introduction of new pharmacokinetic paradigms beyond those that describe the conventional medicines in use today (*see* Chapter 1). With *in vivo* gene transfer, one must account for the fate of the DNA vector itself (volume of distribution, rate f clearance into tissues, *etc.*), as well as for the consequences of altered gene expression and pro t in function. A multicompartmental model t describe these events in a quantitative fashion has been developed

(Ledley and Ledley, 1994). Processes that must be considered include the distribution of the DNA vector following *in vivo* administration; the fraction of vector taken up by the target cell population; the trafficking of the genetic material within cellular organelles; the rate of degradation of the DNA; the level of mRNA produced; the stability of the mRNA produced; the amount and stability of the protein produced; and the protein's compartmentalization within the cell, or its secretory fate, once produced. It is conceivable, although yet to be realized, that each of these events may be incorporated into the design of the gene transfer system in a rational way so as to tailor the gene transfer to the specific requirements of the disease being treated.

Duration of Expression of Transferred Gene. The length of time over which the transferred gene will function is of tremendous importance. In the treatment of inherited diseases, it would be desirable to have stable gene expression over many years. In contrast, in the treatment of malignancy, it is possible that the long-term production of the therapeutic protein could have deleterious consequences. Durable gene expression has yet to be conclusively demonstrated by any of the human trials to date, but this relates as much to the short term of follow-up as to experimental design. Vectors that integrate the transferred DNA into the chromosomes of the recipient cell have the greatest potential for long-term expression. Retroviral vectors and adeno-associated viral vectors have integrating functions. The persistence of the transgene DNA in the DNA of the recipient cell does not, however, guarantee long-term gene expression in that cell. The production of the intended mRNA and protein may decline due to inactivation of the transgene promoter even though the DNA persists. In some circumstances, loss of transgene expression may occur due to loss of the transduced cell by host immune processes (*see* Jolly, 1994, for detailed discussion of this issue).

Adverse Consequences of Heterologous Gene Expression. Along with factors that limit gene transfer and expression, there is a growing list of adverse consequences that may arise as a result of successful gene transfer. As with any new drug, it will be impossible to predict these events in advance of more clinical experience. Nonetheless, some specific events can be anticipated independent of the transgene employed. Because, in most circumstances, gene transfer will result in the synthesis of a new protein, the possibility of an immune response must be considered. A severe immune response could inactivate a secreted product (as is seen in hemophilia patients receiving factor VIII replacement therapy) or lead to an "autoimmune" response to transduced tissues. In some circumstances, the DNA vector itself may be immunogenic, as has been demonstrated for adenovirus vectors. An immune response to the vector may preclude its readministration or limit the duration of its effectiveness.

Pathological events may arise from viral vector replication. Significant efforts have been directed toward the design of viral vectors that are unable to replicate (replication-incompetent) in the target cell. This has been achieved by the deletion of specific genes from the viral genome that are necessary for viral replication (Miller *et al.*, 1993; *see also* legends to Figures 5–1 and 5–2). In order to produce the virus, it must then be grown *in vitro* in a cell specifically designed to provide those functions removed from the virus. By these means, replication-defective retroviruses, adenoviruses, adeno-associated viruses, and herpes viruses have been produced. This approach does not completely eliminate replicative potential in all circumstances. The virus may overcome the deletion of replication machinery by the use of unidentified host cell factors or by recombination in the patient with wild-type viruses. Fortunately, in the limited patient experience to date, these events have not been reported.

Ethical Issues

As with any new technology, much attention has been directed toward ethical issues of gene therapy. Many of these issues are common to all new and expensive forms of medical treatment, such as who will have access to the therapy, and who will pay for it. The perception that this technology could be used for germ-line genetic engineering has spawned much discussion as well (Neel, 1993). Also of concern is the possibility that gene transfer techniques would be used for "frivolous" purposes such as cosmetic alterations. While these issues likely will be topics of continued debate, they, at present, deal with very unlikely events. For example, gene transfer into germ-line tissues to prevent future generations of affected children would require "prophylactic" treatment of prospective parents. Since the risk of having an affected child in the vast majority of cases is either one in two (autosomal dominant disease), or one in four (autosomal recessive disease), and the treatment will be neither without risk nor 100% effective, it is unlikely that any reasonable parent would submit to such a procedure. Even if there were successful introduction of a new gene during the process of *in vitro* fertilization, it is unlikely that the corrected phenotype would persist for more than one generation. The new gene would have to be inserted into the same chromosome (23 to 1 odds against this), and in close proximity to the de-

fective gene (100 to 1 odds against this), so that the new gene would be tightly linked to the defective gene. Alteration f normal characteristics is even more farfetched, as we have only a primitive understanding of the many factors that control physical appearance, personality, intelligence, and physical ability, and the genetic contribution to these characteristics.

TECHNOLOGIES FOR *in vivo* GENE TRANSFER

The ideal DNA delivery system would be one that could accommodate a broad size range of inserted DNA, was available in a concentrated form, was easily produced, could be targeted to specific types of cells, would not permit replication of the DNA, could provide long-term gene expression, and was nontoxic and nonimmunogenic. Such a DNA delivery system does not exist, and none of the available technologies for *in vivo* gene transfer is perfect with respect to any one of these points. As of 1995, three gene transfer systems (retroviral vectors, adenoviral vectors, and liposomes) had been used in human gene therapy trials, with a total clinical experience of a few hundred patients worldwide. Consequently, the following discussion will highlight conceptual strategies and issues to be refined, rather than clinical experience.

Viral Vectors

The natural life cycle of mammalian viruses has made them a logical starting point for the design of therapeutic gene transfer vehicles, because viruses all transfer and express exogenous genetic material during infection. In the simplest analysis, a virus consists of genetic material encapsulated in a particle that can be taken up by the target cell, leading to the expression of virally encoded genes. For viral vectors to be useful, several viral functions must be altered. At a minimum, the virus must be rendered replication-incompetent to prevent uncontrolled spread of the transgene and must have some element of its own genome removed to allow for insertion of the transgene. Beyond this, additional modifications are dependent on the specific virus. Viral vectors have been used extensively in preclinical research and are the basis for the majority f g ne therapy clinical trials now underway.

Retroviruses. Retroviral vectors have had the greatest clinical use so far and ffer the potential for long-term expression from a stably integrated transgene. They lack irrelevant and potentially immunogenic proteins, and there is no preexisting host immunity to the vector. Their application, however, is limited to dividing cells. Large-scale production is technically possible, although purification and concentration potentially are problematic due to the instability of the virus. Several safety issues have been raised but have not as yet been supported by clinical experience.

Retroviruses were first described for gene transfer applications in 1981 and first utilized in clinical trials in 1989 (Rosenberg *et al.*, 1990). Retroviruses are composed of an RNA genome that is packaged in an envelope derived from host cell membrane and viral proteins. For the retrovirus to effect gene expression, it must first reverse transcribe its positive-strand RNA genome into double-stranded DNA, which is then integrated into the host cell DNA. This process is mediated by reverse transcriptase and integrase proteins contained in the retrovirus particle. The integrated provirus is able to use host cell machinery to carry out transcription of viral mRNAs and their subsequent processing and translation into viral proteins. The virus completes its life cycle by synthesizing new positive-strand RNA genomes from the integrated provirus. An encapsidation signal (ψ) within the RNA mediates the organization of the viral genomic RNA and proteins into particles that bud from the cell surface.

Design of the Retroviral Vector. The genomic organization of retroviruses is simple, and this property facilitates its manipulation into vectors for use in gene therapy. The murine leukemia virus and its congeners are the most widely used retroviral vectors (Miller *et al.*, 1993). Retroviral vectors are constructed from the proviral form of the virus. The *gag*, *pol*, and *env* genes are removed to make room for the gene(s) of therapeutic interest and to eliminate the replicative functions of the virus (*see* Figure 5–1 for a strategic overview). Up to 8 kilobases of heterologous DNA can be incorporated into the retroviral vector. Because all virally encoded mRNAs are eliminated from the recombinant retrovirus, no viral proteins are produced by retroviral vectors. This removes any potential viral-encoded antigens that might lead to an immune response to the transduced cells. Along with the gene of therapeutic interest, genes encoding antibiotic resistance often are included in the recombinant retrovirus as a means of selecting the virus-harboring cultured cells *ex vivo*. The bacterial gene for aminoglycoside-3'-phosphotransferase, which confers resistance to kanamycin, neomycin, and geneticin, and the gene for hygromycin B phosphotransferase, which confers resistance to hygromycin, are two such examples of antibiotic resistance genes introduced into retroviral vectors for gene therapy. The presence of an antibiotic resistance gene facilitates isolation of the recombinant retrovirus and subsequent determination of virus titer. Sequences containing promoter and enhancer functions also may be included with the transgene to facilitate its efficient expression and, in some circumstances, to provide for tissue-specific expression after administration *in vivo*. Alternatively, the promoter and enhancer functions contained in the long terminal repeat of the virus may be used for this purpose.

Packaging Cell Lines. After deletion of the genes encoding viral structural proteins and proteins that mediate viral replication, these viruses can be produced only in specially engineered viral packaging cell lines that are capable of providing these proteins (*see* Figure 5–1). The packaging cell line is optimally constructed by stably inserting the deleted viral genes (*gag*, *pol*, and *env*) int the cell in such a manner that these genes will reside on different chromosomes within the packaging cell. This strategy ensures that recom-

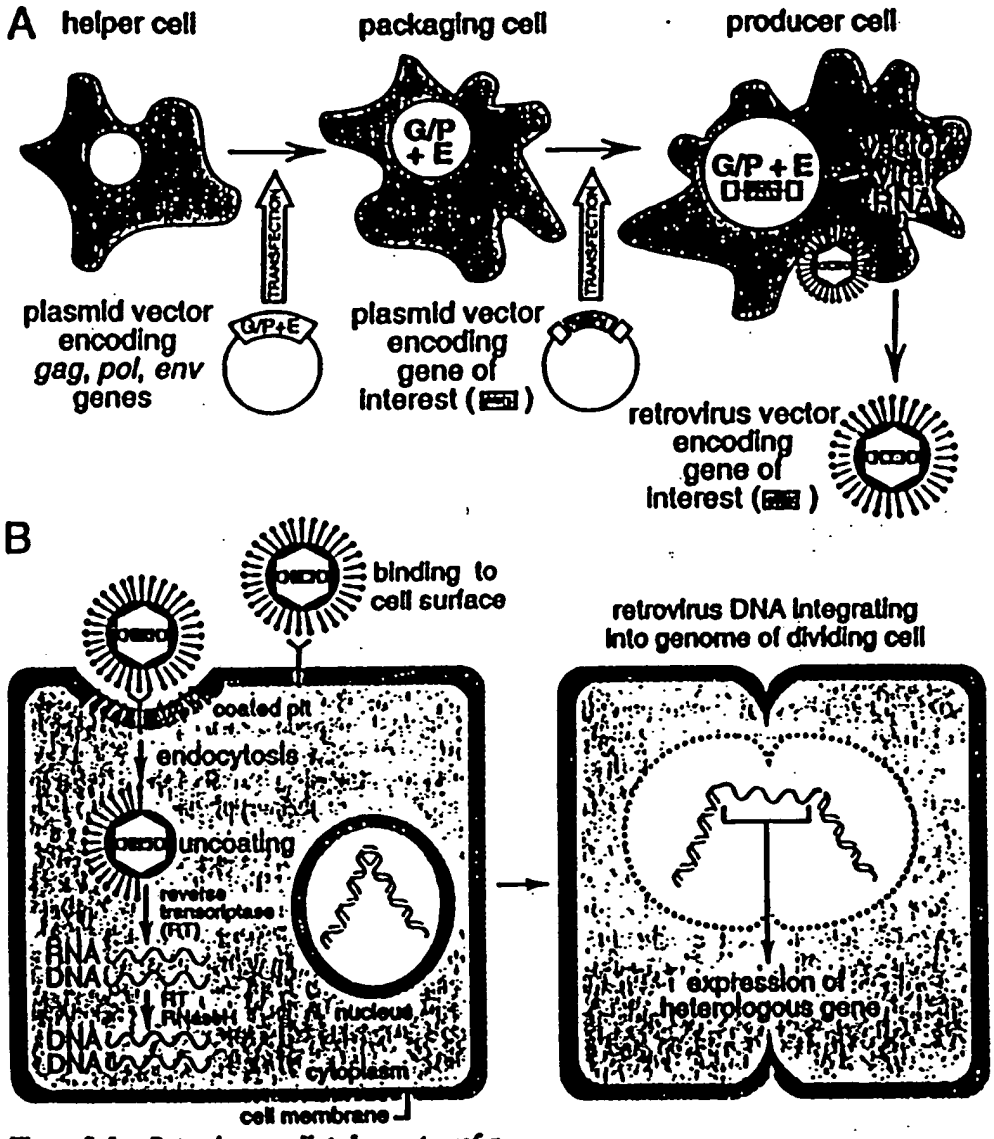


Figure 5–1. Retrovirus-mediated gene transfer.

A. Overall strategy of retroviral production. Replication defective retrovirus vectors are produced from a helper cell that is engineered to provide viral functions (DNA) which have been removed from the virus. The *gag* (G), *pol* (P), and *env* (E) DNA sequences are cloned into DNA plasmids which are then transfected into the helper cell to produce the *packaging cell.* Packaging cells are able to produce the gag, pol, and envelope proteins required for retroviral replication. A plasmid containing recombinant proviral DNA, but lacking *gag, pol,* and *env* genes, is transfected into the packaging cell line to create the *producer cell* which contains all of the molecular machinery necessary to reproduce the recombinant retrovirus that is secreted into the tissue culture medium. Only the recombinant proviral sequence is packaged into the retrovirus. Because the recombinant retrovirus does not contain the *gag, pol,* and *env* genes, cells that this replication-defective recombinant retrovirus infects cannot produce additional virions.

B. Expression of gene of interest in target cell following retrovirus-mediated RNA delivery.

bination of these genes is highly unlikely. In the absence of such a recombination, it is impossible to produce an intact viral genomic RNA that could be packaged into a replication-competent virus. The packaging cell line is used to construct a retroviral producer cell line that will generate replication-defective retrovirus containing the gene(s) of interest. This is done by inserting the recombinant proviral DNA int the packaging cell line. The recombinant proviral DNA is in the form of plasmid DNA containing the long terminal repeat sequences flanking a small portion of the *gag* gene that contains the encapsidation sequence and the genes of interest. This is transfected into the packaging cell line using any number of standard techniques for DNA transfer and uptake (electroporation, calcium precipitation, *etc.*). Several versions of this basic design have been employed to decrease the likelihood of recombinant events that could lead to the production of replication-competent virus (Jolly, 1994). Additional modifications have been employed to alter the host cell range of the virus. This is determined to a large extent by the envelope gene *(env)*. The Moloney murine leukemia virus envelope is ecotropic, which means that infection is restricted to the cells of a particular species, in this case mouse. An envelope affording a broader infection range is available by using the *env* gene from the 4070A strain of murine leukemia virus. This envelope gene has amphotropic specificity and can promote the infection of human, murine, and other mammalian cells. *Env* genes with specificities that extend the host range to nonmammalian cells also are available. Efforts to design new ligands into the envelope protein have met with limited success, as the virus produced often is of low titer. Nonetheless, the ability to specifically target the virus by redesign of its molecular structure is an important goal and undoubtedly will receive more attention in the future.

Clinical Administration of Retroviruses. The clinical administration of retroviruses has been accomplished by the *ex vivo* transduction of patients' cells, by the direct injection of virus into tissue, and by the administration of the retroviral producer cells.

***Ex Vivo* Gene Transfer.** The *ex vivo* approach has been most widely employed in human clinical trials. Although cumbersome in that it requires the isolation and maintenance in tissue culture of the patient's cells, it has the advantage that the extent of gene transfer can be quantified readily and a specific population of cells can be targeted. In addition, a high ratio of viral particles to target cells can be achieved and thus improve the transduction efficiency. This approach was used to modify lymphocytes (Anderson *et al.*, 1990; Rosenberg *et al.*, 1990; Culver *et al.*, 1991) and hematopoietic cells (Nienhuis *et al.*, 1991), in the treatment of adenosine deaminase deficiency (Anderson *et al.*, 1990), in the treatment of hyperlipidemia (Grossman *et al.*, 1994) (*see* Figure 5–4, below), and to express immune modulatory agents in tumor cells (Lotze *et al.*, 1992; Lotze, 1993; Lotze *et al.*, 1994). Clearly, not all potential disease applications are amenable to *ex vivo* gene transfer, as the removal and culture of patient cells may not be technically possible. In such circumstances, direct introduction of the virus *in vivo* is necessary.

***In Vivo* Gene Transfer.** Retroviruses are being tested as potential agents to treat brain tumors which, in many circumstances, are relatively inaccessible. Here, the inherent ability of a retrovirus to transduce only dividing cells (tumor cells) and leave nondividing cells (normal brain parenchyma) untouched may be particularly advantageous. Although the direct stereotactic injection of recombinant retrovirus into the target tissue is possible, the efficiency of gene transfer generally is very low.

Several factors contribute to the inefficiency of retroviral gene transfer *in vivo*. Retrovirus preparations are relatively dilute compared with other vectors, typically with 10^6 to 10^8 plaque forming units per milliliter. Furthermore, the virus can transduce only dividing cells, and within the target tissue only a small fraction of cells may be dividing in the time interval between virus injection and virus clearance. Thus, even with a large excess of virus, only a fraction of the cells are effectively transduced. To overcome these difficulties, Oldfield and colleagues (1993) proposed the administration of a retrovirus producer cell line directly into patients' brain tumors using stereotactic injection. Their hypothesis was that the murine producer cell would survive within the brain tumor for a period of days, and that over this time period would secrete retrovirus capable of transducing the surrounding brain tumor. Studies are in progress in a limited number of patients using virus carrying the herpes virus thymidine kinase gene. This gene renders the cells susceptible to killing by the systemically administered antibiotic ganciclovir, which is metabolized to a cytotoxic metabolite by thymidine kinase. Several important issues will need to be addressed before this approach gains widespread acceptance. The ability of the virus to diffuse from the producer cell to nonneighboring tumor cells is not yet well quantified. If the area of transduced tumor cells is small, tumor cells lying in microscopic cords of tumor infiltrating normal brain might go untreated. Also unknown is whether an immune response to the xenogeneic producer cell line precludes subsequent retreatment of residual tumor. This will be very important given that, over the time of virus secretion, all tumor cells may not be actively dividing, and therefore some cells might go unscathed. Serial treatments, as in conventional chemotherapy, might be required to achieve complete tumor eradication. The results of clinical trials now under way and subsequent studies may answer these questions.

Safety of Retroviral Vector Strategies. The use of retroviral vectors has raised several important safety issues. One concern is that because the virus integrates into the target cell chromosomes (an attractive feature for long-term expression) and because integration occurs in a nearly random fashion, integration could be mutagenic. For example, undesired mutations might occur if insertion of the retroviral DNA altered the function of a cell growth regulating gene. Although replication-competent retroviruses have tumorigenic potential, this has not been observed with the replication-defective vectors that are in use as gene transfer agents. Additionally, this has not been observed in any patients who have received retroviral gene therapy. However, the number of patients studied to date is too few and their follow-up too short for current clinical experience to be extrapolated to long-term safety.

Demonstrating that retroviral agents are free of replication-competent virus is of paramount importance. Replication-competent virus could arise by several means. As noted earlier, recombination of the retroviral genetic elements inserted into the packaging cell line is exceedingly unlikely. Recombination with other retroviral genomes is, however, theoretically possible. There exist homologous endogenous retroviral sequences within the mouse cell lines used to create packaging cell lines. The use of dog- or human-derived packaging cell lines that are free of such sequences has been proposed (Jolly, 1994). Recombination with retroviral sequences in the target cell is theoretically possible. Wild-type murine retroviruses, from which genetic vectors are derived, do not infect human cells. Therefore, it is unlikely that a wild-type virus could infect the same target cell and lead to rescue of the defective retroviral vector. However, there do exist endogenous retroviruses in all human tissue (HERV-K retroviruses)

that have low-level homology to the retroviral vectors. It is very unlikely that this type of recombination would occur with sufficient frequency to lead to clinically significant adverse outcomes. In the final analysis, the safety of these and other vectors must be determined by direct clinical experience and their safety weighed against their therapeutic benefits.

Aden viruses. Over 40 serotypes of human adenoviruses are known, and many animal adenoviruses have been characterized to varying degrees. The clinical spectrum f human adenoviral infections is well described (*see* Horwitz, 1990). Infections involving the respiratory tract are common and typically self-limited in normal hosts. Gastrointestinal, urinary, hepatic, and CNS infections occur sporadically. Most, if not all, adults have prior exposure to adenovirus and are seropositive for antiadenovirus antibodies when tested by sensitive methods. In the United States, military recruits specifically are vaccinated with a polyvalent adenoviral vaccine to prevent outbreaks of respiratory infections (Rubin and Rorke, 1994). In contrast to the retroviruses, these larger, nonenveloped viruses possess a double-stranded DNA genome, and replicate independent of host cell division.

Adenoviral vectors possess several attractive features that have encouraged their development for clinical use. They are capable of transducing a broad spectrum of human tissues, including respiratory epithelium, vascular endothelium, cardiac and skeletal muscle, peripheral and central nervous tissue, hepatocytes, the exocrine pancreas, and many tumor types. Exceptionally high levels of gene transfer and transgene expression can be obtained in dividing and nondividing cells. Several routes of administration can be used including intravenous, intrabilliary, intraperitoneal, intravesicular, intracranial and intrathecal injection, and direct injection of the target organ parenchyma. So far it has not been possible to modify the adenovirus to achieve a tissue-specific virus. The multiple routes of administration may overcome this deficiency by providing flexibility in targeting based on anatomical boundaries.

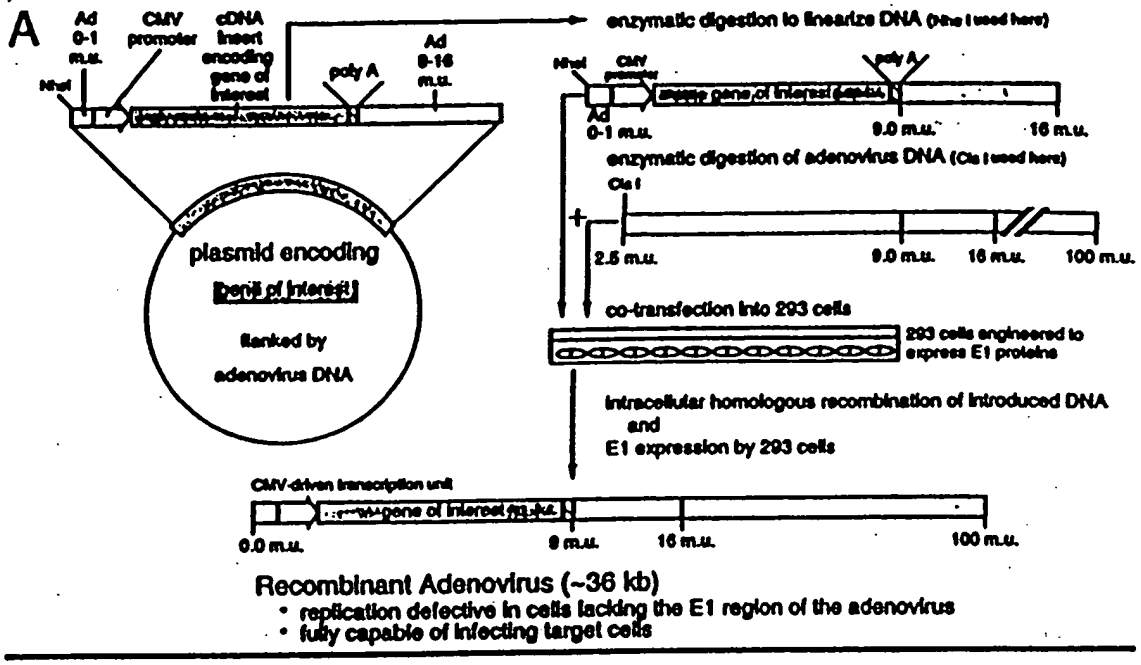
Clinical trials using adenovirus have been limited to date to the ongoing protocols for cystic fibrosis, where the recombinant adenovirus is delivered by aerosolization into the respiratory tract. Studies using direct administration of adenoviral vectors into the liver to treat inherited genetic deficiencies and into a variety of tumors likely will begin in the near future (*see* Ohno *et al.*, 1994, and Kozarsky *et al.*, 1994, as two examples of adenoviral gene therapy strategies).

The genomic structure of adenoviruses is more complex than that of retroviruses. The adenoviral genome encodes approximately 15 proteins. Infection takes place when the fiber protein, which extends from the icosahedral capsid, binds a cell surface receptor. Subsequently, peptide sequences in the penton base portion of the capsid engage integrin receptor domains ($\alpha_v\beta_3$, or $\alpha_v\beta_5$) on the cell surface. This leads to virus internalization via endosomal pathways where the viral particle begins to disassemble. The virus escapes the endosome prior to its fusion with lysosomal compartments and thus avoids digestion. The viral DNA is able to enter the target cell nucleus and begin transcription of viral mRNA without concomitant cell division. Although integration of viral DNA into the host cell genomic DNA can occur at high levels of infection in dividing cells, this is a relatively infrequent event and does not contribute significantly to the utility of these viruses as vectors. Viral gene expression and replication occur in an ordered fashion and are driven in large measure by the E1A and E1B genes in the 5′ portion of the adenoviral genome. The E1A and E1B genes provide transactivation functions for transcription of several of the downstream viral genes (*see* Horwitz, 1990).

Since the E1 genes are involved intimately in adenovirus replication, their removal renders the virus replication-incompetent or, at the very least, severely crippled with respect to replication. Due to the complexity of the virus, it has been more difficult to remove all adenoviral genes as is done with retroviral vectors. The expression of adenoviral proteins, with the currently employed adenoviral vectors, leads to both a cellular and a humoral immune response to recombinant adenoviral vectors. In some instances, this may limit the utility of this vector both in terms of host immune response to adenovirally transduced cells and with respect to readministration of the vector.

Design of Adenoviral Vectors for Gene Therapy. Although several adenoviral serotypes are known, serotypes 2 and 5 have been most extensively used for vector construction. Adenoviral vectors can be constructed using one of several general approaches. A schematic diagram outlining the basic elements of an adenoviral vector design for gene therapy is shown in Figure 5–2. Bett and colleagues (1994) have developed an adenoviral type 5 vector system based on bacterial plasmids containing the adenovirus genome with deletions of the E1 and E3 adenoviral genes. Deletion of E1 renders the virus replication-defective. In addition, all or part of the E3 region, which is not essential for virus function, is deleted in order to accommodate the DNA inserted into the adenovirus genome. Genes of interest can be cloned into the deletion regions, and the plasmid vector can then be grown in bacterial culture. The purified plasmid DNA subsequently is transfected into the 293 line of human embryonic kidney cells. The 293 cell line has been engineered to express E1 proteins and can thus transcomplement the E1-deficient viral genome. The virus can be isolated from 293 cell media and purified by limiting dilution plaque assays (Graham and Prevek, 1991). An alternative approach is to prepare a plasmid containing the gene of interest, flanked by adenovirus DNA sequences. Transfection of this plasmid into 293 cells along with genomic adenovirus DNA with selected deletions (*e.g.*, E3) leads to formation of adenoviral particles with the transgene replacing E1 genes by homologous recombination. It is this strategy that is given in detail in Figure 5–2. Either direct cloning or homologous recombination can be used to produce E1-deleted, replication-defective adenovirus.

Large amounts of the engineered adenoviral vector system can be produced by growing the recombinant virus in 293 cell cultures. The virus is isolated by lysing the infected 293 cells and purifying the crude lysate by cesium chloride density centrifugation, a procedure that not only separates the virus from other tissue culture–derived substances, but also concentrates the virus to very high titers (over 10^{13} particles per ml). The purified virus is remarkably stable in a variety of aqueous buffers, and can be frozen for a prolonged period of time without loss of activity.



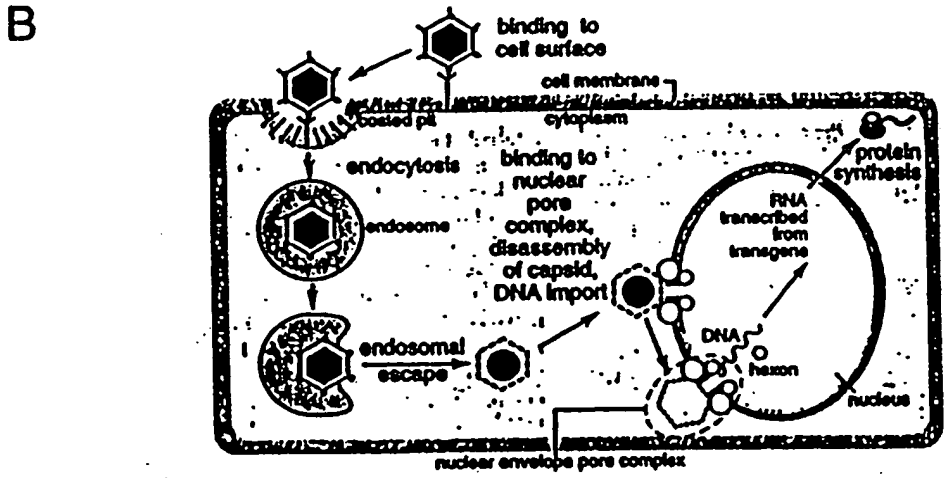


Figure 5–2. Adenovirus-mediated gene transfer

A. Construction of recombinant adenovirus for engineering cells. Strategy for preparing recombinant adenovirus by homologous recombination. Recombinant adenovirus encoding a gene of interest can be produced by cloning the gene of interest (shown in blue) into a plasmid. This transgene is flanked by a promoter sequence (*e.g.*, CMV promoter) and by regions of the adenovirus genome (shown in gray). The example here is based on adenovirus 5. The adenovirus 5 DNA is divided into 100 map units (m.u.; 360 base pairs per map unit). Deletions are made in the adenovirus DNA to remove E1 (1 to 9.2 m.u.) and E3 (78.4 to 84.3 m.u.) regions to eliminate the possibility of autonomous replication and to allow room for insertion of the transgene. Homologous recombination takes place between the plasmid DNA and the adenovirus genomic DNA to yield the recombinant virus. Since the transgene sequence replaces the E1 genes of the adenovirus, the adenovirus is unable to replicate in cells other than those engineered to express E1 gene products, such as the human embryonic kidney 293 cells shown here.

After linearization of the plasmid by digestion with an endonuclease (*e.g.*, Nhe I in this example), the transgene-expressing plasmid is cotransfected with adenovirus genomic DNA from which the 5' end has been removed (*e.g.*, digestion with the Cla I endonuclease at Ad 2.5 m.u.), also to prevent autonomous adenovirus replication, until homologous recombination takes place, which in this example occurs within the 293 cells.

B. Adenovirus-mediated infection of target cells. Expression of gene of interest in target cell following adenovirus-mediated DNA delivery. A recombinant adenovirus binds to specific receptors on the surface of a target cell and enters the cell by endocytosis. Viral proteins promote the escape of the adenovirus from the endosome prior to endosome fusion with and destruction by lysosomes. The adenovirus DNA becomes unpackaged from the viral proteins and travels to the nucleus where it begins to synthesize new mRNA. The adenovirus encoded DNA, including the transgene, is not integrated into the genome of the host cell. (*Modified from Greber et al., 1993, with permission.*)

Duration of Transgene Expression. Adenoviral vectors currently are limited by their relatively short duration of transgene expression. Several factors contribute to this, including clearance of transduced cells by cytotoxic T cells and other inflammatory cells (Yang *et al.*, 1994) and dilutional loss of episomal DNA during target cell division. The former likely will be solved by the design of adenoviral vectors that are less immunogenic. Vectors with temperature-sensitive mutations in the E2 region clearly are less immunogenic and offer significantly longer gene expression (Engelhardt *et al.*, 1994). Deleting the E4 gene from adenoviral vectors also may diminish the immune response to transduced cells (Armentano *et al.*, 1994). Subsequent generations of adenoviral vectors with additional modifications of the adenoviral genome or the use of nonhuman adenoviruses may advance the use of adenoviral vectors. The episomal nature of the adenovirus genome ultimately limits the duration of gene expression in tissues with active cell division such as bone marrow and epithelial surfaces. Since each round of target cell division after gene transfer is not accompanied by replication of the transgene, daughter cells will have progressively fewer and eventually no copies of the transgene. Integration of the adenoviral vector does occur, but not at a high enough frequency to be useful.

Safety of Adenoviral Vector Strategies. The safety of adenoviral vectors likely will be borne out by current clinical trials. The principal side effects are from the host immune response to the adenoviral proteins, a limitation that may be eliminated by future generations of vectors. There is some concern, however, that vector replication can take place despite removal of important regulatory genes. Since wild-type adenoviral infections are common, there exists the possibility that wild-type viruses may recombine with replication-defective vectors to produce replication-competent, recombinant virus. Although not observed in the present cystic fibrosis clinical trials, this remains a concern. Additionally, there is a growing body of evidence that some cell types may contain proteins with functions homologous to E1a and thus be able to provide a permissive environment for recombinant viral replication. With the present adenoviral vectors, this is not likely to evolve into a serious infection, given the preexisting host immunity to adenoviral infection. However, if future adenoviral vectors are able to evade this protective mechanism, then recombinant viral replication may become a greater concern.

Adeno-Associated Virus. Adeno-associated virus (AAV) appears to have many of the desirable features of retroviruses and adenovirus without some of their potential drawbacks for application to gene therapy (Kotin, 1994). These single-stranded DNA, nonautonomous parvoviruses are able to integrate efficiently into the genome of nondividing cells of a very broad host range. Integration of the wild-type virus is specific for chromosome 19 (19q13.3-qter), or at least shows preferential integration at this site. Although ubiquitous in nature, AAV has not been shown to be associated with any known human disease and does not elicit an immune response in an infected human host. AAV is a nonenveloped virus that is stable to a variety of chemical and physical manipulations and thus can be purified, concentrated, and stored for prolonged periods.

At present, the use of AAV as a vector for gene therapy is limited by difficulties in producing the virus in large quantities and, more importantly, by a lack of understanding of the biology of the recombinant virus. For instance, it remains to be determined whether or not these vectors have the ability to infect and integrate into nondividing cells, an important feature of the wild-type virus that has promoted its use. There is little experience in human beings with these new vectors. The Recombinant DNA Advisory Committee of the National Institutes of Health has approved the first human trial of AAV in patients with cystic fibrosis. This trial may provide information about the duration of gene expression following AAV-mediated gene transfer into terminally differentiated airway epithelial cells.

AAV has two distinct phases to its life cycle. In the absence of helper virus (adenovirus), the wild-type virus will infect a host cell, integrate into the host cell genome, and remain latent for a long time. In the presence of adenovirus, the lytic phase of the virus is induced, which is dependent on the expression of early adenoviral genes, and leads to active virus replication. Structurally, the AAV genome is composed of two open reading frames (called *rep* and *cap*) flanked by inverted terminal repeat (ITRs) sequences. The *rep* region encodes four proteins which mediate AAV replication, viral DNA transcription, and endonuclease functions used in host genome integration. The *rep* genes are the only AAV sequences required for viral replication. The *cap* sequence encodes structural proteins that form the viral capsid. The ITRs contain the viral origins of replication, provide encapsidation signals, and participate in viral DNA integration. The function of many of these proteins and the overall biology of the virus have been studied largely in wild-type viruses (*see* Kotin, 1994). Recombinant, replication-defective viruses that have been developed for gene therapy lack *rep* and *cap* sequences. The recombinant viruses are less well studied, and it is not known whether these viruses retain all of the features of the wild-type virus (*i.e.*, site-specific integration in a nondividing cell).

Production of AAV in large quantities is considerably more difficult than production of retroviruses or adenoviruses. Replication-defective AAV can be produced by cotransfecting the separated elements necessary for AAV replication into a permissive cell line (typically 293 cells). In a commonly used method, plasmid DNA containing *rep* and *cap*, under the control of AAV promoters but lacking ITRs, is transfected into 293 cells. DNA containing the gene to be "packaged" (promoter, enhancer, transgene, polyadenylation signal) flanked by ITRs is cotransfected at the same time. Infection with adenovirus provides helper functions that induce the synthesis of rep proteins, which in turn transactivate the synthesis of capsid proteins. The transgene flanked by ITRs is then packaged into viral particles that can be isolated and purified by cesium chloride density centrifugation. This approach requires that the plasmid expressing the ITR (ITR^+; here, the transgene-encoding plasmid) have little sequence homology with ITR^- plasmids (*cap* and *rep*) to reduce the likelihood of recombination events that could lead to the production of wild-type virus. Improved systems for recombinant AAV preparation are being developed including the use of producer cell lines that provide rep and cap functions. Such an approach not only would simplify the transfection scheme, but also would provide rep and cap proteins in larger quantities and thus lead to higher yields of recombinant virus.

Vaccinia Vectors (Pox Viruses). The extensive clinical experience with vaccinia vaccines and their ease of ma-

nipulation have led t efforts t develop gene therapy vectors from pox viruses (Moss and Flexner, 1987; Moss, 1990). Vaccinia are large, enveloped DNA viruses that replicate in the cytoplasm f infected cells. Like adenovirus, they can infect nondividing as well as dividing cells from many different tissues, and provide short-term gene expression from a nonintegrated viral genome. Recombinant virus can be produced by inserting the transgene into a vaccinia-derived plasmid and transfecting this DNA into vaccinia-infected cells. Homologous recombination leads to the generation of the recombined virus that can be plaque purified. High yields of virus are achieved easily and can be stored for long periods of time. The vaccinia viruses can accommodate much larger DNA inserts than can retrovirus, adenovirus, or AAV vectors. Additionally, since the wild-type virus no longer exists in the wild, recombination to produce new strains of virus is unlikely. A significant drawback to the use of this vector system is that it elicits a host immune response to the 150 to 200 virally encoded proteins. This is likely to make repeated administration problematic. Replication of the vector also is a concern, as it can result in significant morbidity in immunodeficient hosts. This might be overcome with newer generations of engineered vaccinia virus. At present, this vector system has not been adopted for clinical trials f human gene therapy, although it may be useful as a vaccine vector.

Herpes Simplex Virus-1 Vectors. The herpes simplex virus is a large (152 kb), double-stranded DNA virus that replicates in the nucleus of infected cells. It has a broad host cell range, and can infect dividing and nondividing cells as well as persist in a nonintegrated state. Large sequences f foreign DNA can be inserted into the viral genome by homologous recombination, and the replication-defective, recombinant virus can be plaque purified on transcomplementing cells (IE^+). These advantages for gene therapy strategies are countered by the difficulty in rendering viral preparations totally free of replication-competent virus and the elicitation of a potent immune response to virus-encoded proteins that are directly toxic to the cell. Despite these apparent drawbacks, advantages such as their ability to accommodate large DNA inserts (20 to 30 kb), the availability of high titer stocks, and their neurotropism have stimulated interest in developing useful herpes virus vectors (*see* Kennedy and Steiner, 1993).

Deletion of the viral thymidine kinase gene renders the herpes virus replication-defective in cells with low levels of endogenous thymidine kinase (*i.e.*, terminally differentiated, nondividing cells). In contrast, cells undergoing active cell division (*e.g.*, tumor cells) possess sufficient thymidine kinase activity to allow the thymidine kinase–negative herpes virus to replicate. This type of vector may be useful for treating intracranial tumors, as the tumor cells, but not the neurons, will selectively undergo gene transfer. Since vector replication occurs, systemic dissemination potentially can occur with this viral vector. This is much less likely in immunocompetent hosts because the host cellular immune response likely will control the spread of the virus. The use of herpes virus vectors in immunocompromised hosts, which may include some cancer patients, is potentially problematic (*see* Valyi-Nagy *et al.*, 1994).

Other Viral Vectors. The need for tissue-selective gene transfer has led to the consideration of a variety of other viruses, including HIV, the minute virus of mice, hepatitis B virus, and influenza virus, as possible vectors for gene transfer. These and other viruses may find applications based on aspects of their life cycle that result in tissue-selective gene expression or other unique features that lend themselves to specific diseases (*see* Jolly, 1994).

Comparison of Properties of Viral Vectors for Gene Therapy. Boviatsis and colleagues (1994) recently compared the usefulness of recombinant retrovirus, adenovirus, and herpes virus vectors in a rat brain tumor model using the gene coding for bacterial β-galactosidase as an indicator of gene transfer. Although their experiments did not definitely establish which vector is more efficient at gene transfer, useful distinguishing features of each vector were nonetheless noted. Following intralesional administration, the retrovirus and herpes virus vectors selectively effected gene transfer into tumor cells over neurons and other endogenous brain cells. In contrast, the adenoviral vector transduced brain tumor cells as well as neighboring normal brain parenchyma. In the case of the retroviral vector, selectivity for the tumor cell results from the virus's requirement for cell division as a prerequisite for transgene integration and expression. In the case of the herpes virus vector, the selectivity occurs as a result of differential expression of endogenous thymidine kinase in the tumor cells (very high) versus nonneoplastic cells (very low). The adenovirus showed little cell selectivity, and any preference for tumor cell expression probably was a result of the site of injection (within the tumor). Another noteworthy observation was the degree of inflammation and necrosis that occurred following gene transfer. The retroviral vector induced no significant inflammatory response, and that induced by the adenoviral vector was minimal. However, prominent inflammatory infiltrates were noted in the brain tissues following herpes virus–mediated gene transfer. Although this study suggests a useful role for the herpes virus vector in treating tumors, the clinical application of such a vector likely will be difficult. Additional measures to control replication of this human pathogen-derived vector will have to be instituted, and the consequences of a potentially

ere inflammatory response will need to be addressed. thermore, as Bovlatsis and colleagues (1994) point out, e latency f this type f vector is unknown, and it is therexe possible that reactivation by recombination with wild-e virus (thymidine kinase positive) could occur.

nviral DNA Delivery Strategies

cause of the potential limitations of viral vectors, inestigators have examined the use of nonviral agents to diate cellular uptake of exogenous DNA. These DNA ivery systems, which include uncomplexed plasmid NA, DNA-liposome complexes, DNA-protein comlexes, and DNA-coated gold particles, are constructed m known components. Therefore, their composition, unxe complex virions, is well-defined. In addition, their forulation technically is much easier than that of viruses l, in many cases, these DNA delivery systems can be oduced without the need for cell culture.

rified Uncomplexed Plasmid DNA. Surprisingly, purified DNA mRNA) can be injected directly into tissues and results in trant gene expression. This has been best illustrated in muscle tissue, here direct injection of uncomplexed DNA is most effective. Wolff *t al.* (1990) demonstrated that purified plasmid DNA or mRNA ending a reporter gene could mediate transgene expression followdirect injection into the quadriceps muscle of a mouse. DNA injction resulted in longer gene expression (substantial gene product as seen after 60 days) than did mRNA injection (expression ded after 18 hours). The DNA likely persists as unintegrated plasd DNA rather than in an integrated form. A direct comparison of denoviral and retroviral vectors with injected plasmid DNA in urine muscle gene transfer revealed that all three systems were more ficient at gene transfer in regenerating muscle (cardiotoxin-inced) than in mature normal mouse muscle. In regenerating mus, these DNA transfer systems were equally efficient, as assessed by the number of muscle fibers expressing the reporter gene. Surisingly, in mature fibers, gene transfer by direct injection of plasd DNA was superior to that with either of the viral vectors (Davis *al.*, 1993). In ddition, no inflammatory response was seen following direct DNA injection, whereas mild inflammation was seen ith either viral vector. To date, direct injection of plasmid DNA has en shown to be highly effective only in skeletal and cardiac muse. Its effectiveness may depend on features unique to the muscle fiber.

NA-Coated Gold Particles. Plasmid DNA can be affixed to gold rticles (approximately 1 micron in diameter) and then "shot" into superficial cells. The DNA is coprecipitated onto the gold particle and then propelled from a mylar sheet using an electric spark or presrized gas as the motive force. This so-called gene-gun can be used accelerate the DNA-coated particles into superficial cells of the kin (epidermis) or into skin tumors (melanomas). Gene expression lasts only a few days, which may be more a function of the cells tarcted (*e.g.*, skin cells that are sloughed) than the method of delivery. animal models, gene-gun delivery of DNA vaccines is highly effective (Fynan *et al.*, 1993). Gene-gun delivery is ideally suited to gene-mediated immunization, where only brief expression of antigen is necessary to achieve an immune response.

Because of the limited depth of DNA penetration, this technique is limited to surface cells that can be accessed directly. Furthermore, since the epidermal layers of the skin are rich in antigen-presenting cells, they are a preferred target for vaccination. The simplicity, safety, and technical ease of preparation of this DNA transfer system make its large-scale application more feasible than available viral DNA delivery systems.

Liposomes. Liposomes have been used extensively as a technology for delivering drugs experimentally to the interior of cells. The premise is that by surrounding hydrophilic molecules with hydrophobic molecules, agents otherwise impermeable to cell membranes might be escorted into the cell. Potential advantages of such a delivery system include targeting drugs to an intracellular location and reducing toxicity.

The basic challenge in *in vivo* gene therapy is to deliver a transgene, a large hydrophilic molecule, across the plasma membrane and into the nucleus where it can access the cell's transcription machinery. Liposome delivery technology appears well suited to this task, although it has not proven to be as efficient as hoped.

Liposomes are either unilamellar or multilamellar spheres that are manufactured using a variety of lipids. Their structure can be influenced by choice of lipid composition and manufacturing process. Proteins and other nonlipid molecules can be incorporated into the lipid membranes. For convenience, liposomes are classified as either anionic or cationic, based on their net negative or positive charge, respectively.

Anionic Liposomes. The first *in vivo* delivery of genes using liposomes was reported by Nicolau and colleagues (1983), who encapsulated a DNA transgene coding for insulin into anionic liposomes and injected the complex into rats. The transfected rats had increased circulating levels of insulin and decreased blood glucose concentrations.

In spite of this early success, there are significant drawbacks to the use of anionic liposomes for delivering DNA. These structures, when given intravenously, primarily target the reticuloendothelial cells of the liver, making them of little use for other cell targets. Because the substance to be delivered must be encapsulated within the liposomes, the manufacturing process is complex. Also, most DNA constructions necessary for gene therapy are large compared with the liposome, so that encapsulation efficiency is very low, probably prohibitively so for practical applications.

Various proteins can be inserted into the external layer of liposomes to alter their *in vivo* behavior, including cell-selective delivery. This approach can enable liposomes given intravenously to evade the reticuloendothelial system. Protein ligands or antibodies to cell surface molecules incorporated into the liposome surface also can target liposomes to specific cell surface receptors on desired cell populations (Wu and Wu, 1987). Although promising, these strategies have not yet been applied successfully to gene therapy.

Cationic Liposomes. Felgner and co-workers (1987) synthesized cationic liposomes and demonstrated that they would avidly and efficiently bind nucleic acids (which are anionic) by electrostatic interactions upon simple incubation of liposomes with nucleic acids at room temperature for brief periods. The DNA or RNA complexed to cationic liposomes readily entered cells in culture without perceptible injury to the cells. A diagram illustrating the presumed mechanism for cationic liposome-plasmid transfection is given in Figure 5–3.

In vivo, cationic liposomes have properties quite different from those of anionic liposomes. Intravenous injection of cationic complexes has been shown to effect transgene expression in most organs if the liposome-DNA complex is injected into the afferent blood supply to the organ. In addition, the liposome-DNA complexes can be administered by intraairway injection or aerosol to target lung epithelium. In experimental animals, neither intravenous injection nor aerosol delivery of cationic liposome-plasmid complexes appears to be toxic (Brigham *et al.*, 1989).

Cationic liposomes have been used to deliver DNA gene constructs in several experimental models *in vivo*. Nabel and colleagues (1994) delivered a foreign histocompatibility gene by direct injection of plasmid-liposome complexes into tumors and showed attenuation of tumor growth in murine models. Hyde and associates (1993) showed that cationic liposome-mediated gene transfer could correct CFTR-dependent, cyclic AMP stimulated chloride conductance to normal levels in transgenic mice homozygous for a null mutation in CFTR. Rabbits given intravenously the gene coding for the proximal enzyme in prostanoid synthesis (prostaglandin synthase) as a plasmid-cationic liposome complex produced increased amounts of endothelium-derived prostanoids in their lungs. This protected the lungs of the transfected animals from the effects of endotoxemia (Conary *et al.*, 1994).

Table 5–1 includes therapeutic goals in early stages of human application using liposome-mediated DNA delivery for gene therapy, such as delivery of foreign histocompatibility gene to tumors, delivery of the human α_1-antitrypsin gene to the nasal mucosa of α_1-antitrypsin-deficient patients and to subsegments of the lungs by fiberoptic bronchoscopy, and delivery of the CFTR gene to the nasal mucosa of patients with cystic fibrosis.

At present, liposome-mediated transfection offers a nontoxic, nonimmunogenic means to deliver DNA to a variety of tissues. Current usefulness of this strategy is limited by generally lower levels of gene transfer than can be obtained with viral vectors, although newer liposome formulations offer improved gene transfer efficiencies and better physical properties, *e.g.*, higher concentrations of complex without aggregation. The applications for liposomes in gene therapy likely will expand as better reagents are developed, particularly those that facilitate targeting of specific cells.

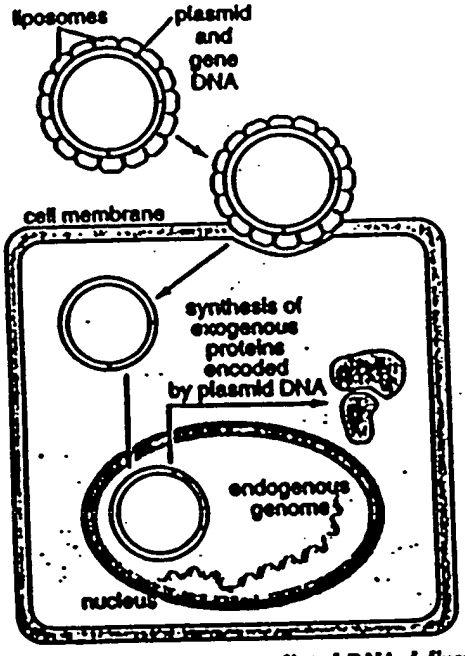


Figure 5–3. Cationic liposome-mediated DNA-delivery.

Diagrammatic representation of how cationic liposome-plasmid complexes are thought to effect gene transfer to a cell. Little is known about the actual structure of the plasmid-liposome complex. Likewise, processes affecting cell entry and transport to the nucleus are yet to be clarified. The circular plasmid DNA does not readily incorporate into the host genome and does not replicate in mammalian cells; thus transgene expression apparently is episomal in nature.

DNA-Protein Conjugates. Several groups have developed cell-specific DNA-delivery systems that utilize unique cell surface receptors on the target cell (Michael and Curiel, 1994). By attaching the ligand recognized by such a receptor to the transgene DNA, the DNA-ligand complex becomes selectively bound and internalized into the target cell (Wu and Wu, 1987). These molecular conjugate vectors are attractive because they potentially offer cell-specific gene transfer without the attendant problems of viral vectors, such as replication, immunogenic viral proteins, or recombination potential. Initial model systems focused on developing effective means of attaching the DNA to the ligand using polycations, antibody-antigen complexes, and biotin-streptavidin linkers. Poly-L-lysine (PLL), a polycation, has been widely used as it can be easily coupled to a variety of protein ligands by chemical cross-linking methods. When the PLL-ligand adduct is mixed with plasmid DNA, macromolecular complexes form in which the DNA is electrostatically bound to the PLL-ligand molecules. These toroidal structures (50 to 100 nm in diameter) present ligands to the cell surface receptor that are efficiently endocytosed. The transferrin receptor (Zenke *et al.*, 1990), the asialoorosomucoid receptor (Wu and Wu, 1987), and cell surface carbohydrates (Batra *et al.*, 1994) have been used to demonstrate the potential of ligand-mediated gene delivery. The asialo-orosomucoid receptor is of particular interest because it is found almost exclusively on hepatocytes and therefore might be useful in mediating gene transfer into the liver.

Early DNA-ligand complexes were inefficient for DNA transfer because most of the endocytosed complex was shuttled to the lysosomal compartment, and DNA was

then degraded. Although several agents (*e.g.*, chloroquine) have been used t block lysosomal degradation, the efficiency f transfection is still low compared with other DNA-delivery methods. A more ffective approach is to utilize the endosomal escape functions f the adenovirus. As described earlier, proteins in the adenovirus capsid promote escape of the DNA complex from the endosome prior t fusion with the lysosome. Although metabolically inactivated adenovirus theoretically could be employed to escape lysosomal targeting, the concentrations of adenovirus required to ensure colocalization of the virus and the DNA-protein complex to the same endosome are so high as to induce adenovirus-mediated cytopathic effects. Consequently, investigators have constructed physically linked complexes between the adenovirus and the DNA-ligand adduct, thereby ensuring their simultaneous delivery to each endosome and diminishing the amount of adenovirus required to escape lysosomal delivery and degradation (*see* Fisher and Wilson, 1994).

Two general approaches have been used to construct adenovirus-DNA-ligand complexes. Poly-L-lysine can be covalently attached to purified adenoviral particles using a water-soluble carbodiimide. This is then mixed with asialo-orosomucoid receptor–poly-L-lysine–DNA toroids to form clusters of icosahedral adenoviral particles and toroids. The size of these clusters varies from small clusters (<200 nm) with single toroids coupled to two viral particles up to large clusters (200 to 300 nm) containing more than a dozen viral particles and toroids. The composition of the clusters is governed by the amount of poly-L-lysine attached to the viral particles. These complexes achieve higher levels of hepatocyte-specific gene transfer at lower concentrations of virus than do mixtures of unlinked toroids and adenovirus (*see* Cristiano *et al.*, 1993).

This technology can be further improved by layering the DNA and ligand over the surface of the adenovirus to create a coated adenovirus, rather than the side-by-side (virus-toroid-virus) structures described above (Fisher and Wilson, 1994). This creates single viral particles that retain their endosomalysis ability, are coated with DNA and extend the asialo-orosomucoid receptor from the particle surface. These smaller particles (<100 nm) still retain some adenovirus receptor recognition and uptake, similar to the larger clusters above, but their smaller size may make them better able to traverse the fenestrated hepatic endothelium. The use of two reporter genes, one carried in the plasmid DNA and the other in the adenovirus genome, has allowed the simultaneous assessment of viral infectivity and efficiency of plasmid gene transfer. By decreasing the amount of adenovirus required, virus-induced cytotoxicity essentially can be eliminated. The presence of two receptor pathways for DNA entry (ligand receptor and adenovirus receptor) clearly diminishes the specificity of this DNA delivery system. The adenovirus receptor pathway can be effectively eliminated by using an antibody against adenovirus fiber protein as the means for linkage to DNA (Michael and Curiel, 1994), an approach that obliterates the ability of the virus to bind adenovirus receptors but not its ability to mediate lysosomal escape. Further refinements, such as the use of purified endosomalytic proteins rather than intact adenovirus particles, should enhance the utility of this type of DNA-delivery system (Seth, 1994).

DISEASE TARGETS FOR GENE THERAPY

Organ-Directed Gene Therapy

Liver. Liver-directed gene therapy has emerged as an important model for the treatment f inherited and acquired disorders. The liver can be afflicted with a variety of metabolic, infectious, and neoplastic diseases for which specific molecular interventions can be envisioned. For example, gene transfer methods might be used to deliver interferon alfa for the treatment of hepatitis B, cytotoxic therapy for hepatic carcinomas, or to provide a missing gene to correct an inherited metabolic defect. Potential applications are made more feasible by the existence of multiple methods for targeting gene transfer to the liver. Molecular conjugates, adenoviral vectors, liposomes, and retroviral vectors all have been used for hepatocyte gene transfer. For *in vivo* gene transfer, the liver is accessible by a number of routes, including direct injection and intravenous and intrabiliary administration of vectors. *Ex vivo* strategies can be implemented by partial surgical resection of the liver, isolation of hepatocytes, and *in vitro* hepatocyte transduction. The genetically modified cells can be reimplanted into the liver.

Familial Hypercholesterolemia. Patients with familial hypercholesterolemia have an inherited deficiency of the low-density lipoprotein (LDL) receptor and, as a consequence, develop extremely high plasma levels of cholesterol and arteriosclerosis at a very early age (*see* Chapter 36). The genetic defect manifests itself as a diminished ability of the liver to clear LDL particles from the blood, and serum lipid levels provide a convenient marker of the disease. Although pharmacological interventions have had limited success, correction of the hepatic dysfunction by orthotopic liver transplantation leads to normalization of blood lipid levels and slowing of arterial disease progression. This clinical observation suggested that if the liver could be genetically modified to express the LDL receptor, the same benefits might be achieved. The Watanabe heritable hyperlipidemic rabbit has served as an ideal animal model to demonstrate that this approach could lead to persistent reductions in serum LDL (*see* Figure 5–4) (Chowdhury *et al.*, 1991). Several patients now have been treated in a clinical trial using an *ex vivo* DNA delivery approach and retrovirus to introduce the LDL receptor gene into hepatocytes isolated from the patients following partial hepatectomy (Grossman *et al.*, 1994). This study demonstrated the feasibility, safety, and potential efficacy of *ex vivo* hepatic gene therapy.

The overall success of DNA transfer into hepatocytes will be determined by several factors that currently are unknown. In particular, very little is known about the normal turnover of hepatocytes and how this will relate to the persistence of genetically modified cells. An immune response to the therapeutic gene product, a potential problem for all gene therapies of deficiency states, has not been observed to date. The potential for the therapeutic gene product to serve as a neoantigen may vary among different types of deficiencies and depend on the nature of the protein product and whether the deficiency arises from total absence of the protein or from expres-

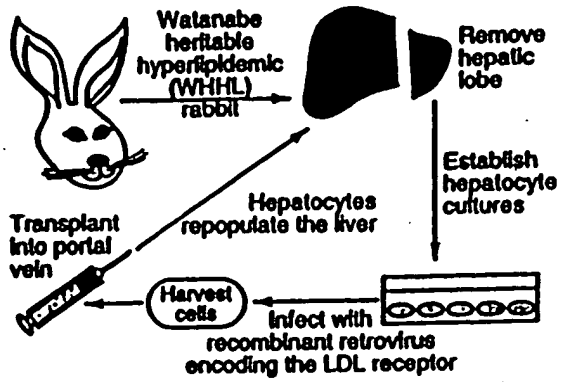


***Figure 5–4** An animal model for ex vivo retrovirus gene transfer of the low density lipoprotein (LDL) receptor.*

The Watanabe heritable hyperlipidemic (WHHL) rabbit is an ideal animal model of the inherited deficiency in the LDL receptor. Lacking the LDL receptor normally expressed in hepatocytes, these animals rapidly develop atherosclerosis. The feasibility of *ex vivo* retrovirus gene transfer is demonstrated by this model. A partial hepatectomy is performed, removing up to one-third of the liver. The resected portion of the liver is perfused *ex vivo* with enzymes to disperse the hepatocytes, which are then placed in tissue culture and exposed to recombinant retrovirus expressing the LDL receptor. Hepatocytes containing the stably integrated viral DNA are injected through the portal vein back into the liver where they take up residence. This procedure now has been conducted in human patients with the same disorder.

sion of a dysfunctional (mutated) protein. The clinical trial cited above (Grossman *et al.*, 1994) provides the first example of sustained metabolic correction of a genetic defect. The *ex vivo* gene transfer approach likely will be replaced by *in vivo* gene transfer strategies in the future, once problems of vector efficacy, persistence, and immunogenicity are overcome.

Lung. The two most common inherited lung diseases are familial emphysema and cystic fibrosis. Gene therapy strategies have been directed toward the amelioration of both these diseases.

Familial Emphysema. Familial emphysema is a consequence of a defect in the gene encoding the principal endogenous antiprotease, α_1-antitrypsin. This deficiency renders the lungs vulnerable to injury by neutrophil proteases released at sites of inflammation. The α_1-antitrypsin protein is available clinically and is given to patients with the disease. The human gene has been cloned and delivered effectively to the lungs of experimental animals (Canonico *et al.*, 1994). Initial studies in human beings with α_1-antitrypsin deficiency have been approved by the NIH (*see* Table 5–1).

Cystic Fibrosis. Cystic fibrosis is the most common inherited disorder in the Caucasian population, and because most of its morbidity and mortality stems from pulmonary manifestations, it is an ideal model for gene therapy of inherited lung disease. *Ex vivo* gene transfer strategies are not a viable option in the lung. Removal and reimplantation of airway cells is not technically feasible for therapy. Because the target cells in the airway turn over very slowly, retroviral gene transfer, which requires cell division, is very inefficient. In contrast, adenoviral vectors are uniquely suited for this application, as adenovirus has a known tropism for respiratory epithelium. A major potential drawback to the use of adenovirus is the transient nature of gene expression and uncertainty as to whether an adenovirus-induced inflammatory response will allow readministration of the vector. Additionally, airway neutrophils and secretions may decrease transfection efficiency. Nonetheless, a major effort has been launched to develop adenoviral vectors suitable for transducing airway epithelia *in vivo*.

Human studies have been conducted in which adenovirus encoding the cystic fibrosis transport regulator (CFTR) was administered into the nasal epithelium of patients with cystic fibrosis (Zabner *et al.*, 1993). With relatively low doses of virus, normalization of chloride conductance was observed. The major current disadvantage of adenovirus as a vector has been the host response to virally encoded proteins. An inflammatory response to adenovirally transduced cells has been observed in a variety of animal models and in patients, because the vector contains most of the wild-type viral genome. Although the virus has been rendered replication-incompetent by deletion of a subset of viral genes, it still directs the virally transduced cell to synthesize immunogenic viral proteins. Newer versions of the recombinant adenoviral vector may overcome this limitation by attenuating the expression of adenoviral proteins. Engelhardt and colleagues (1994) have shown that alterations of the adenoviral genome in addition to E1 and E3 deletions can decrease the inflammatory response following gene transfer. A temperature-sensitive E2 mutant (ts 125) that preferentially grows at 32° C is introduced into the viral genome so that, when the virus is used to infect cells at 39° C, the mutant E2 protein is less effective in transactivating downstream adenoviral genes that presumably are responsible for inducing the host inflammatory response. In practice, the virus can be propagated in permissive cells (293 cells) at 32° C *in vitro*, and then used to transduce cells *in vivo* at 37° C. Following *in vivo* transduction, the virus is replication-defective (E1 deleted) and less efficient in the synthesis of adenovirus proteins at the elevated body temperature. This results in less inflammation and prolonged transgene expression. Further improvement in the design of adenoviral vectors is under development, including mutations that will remove all or part of the E4 region.

At present, the number of patients treated in all cystic fibrosis gene therapy trials is too small to draw any meaningful conclusions as to efficacy. However, the principles of airway delivery of genetic material are now well established. Future generations of genetic DNA transfer systems, including the adeno-associated virus and liposome systems discussed earlier, likely will offer meaningful benefits not only for cystic fibrosis but also for a variety of lung disorders.

Vasculature. The blood vascular system has been the target of several gene transfer experiments that have demon-

ted the therapeutic potential of gene delivery into this e. Both the endothelial cells that line the blood ves- and the smooth muscle cells beneath the endothelium ve attracted much attention because f their role in ath- erosclerosis and the prospect that they might be used to er transgene products into the bloodstream. Genetic erations of these cells might be useful to alter or pre- the process of atherosclerosis, or to deliver vasodilat- gents locally or, alternatively, to provide local deliv- of anticoagulants.

vo Strategies. Initial experiments focused on *ex vivo* gene er methods. Wilson *et al.* (1989) demonstrated that canine en- elial cells could be genetically modified *in vitro* by retroviral e transfer and then transplanted back into the dog as a Dacron® lar implant seeded with the modified endothelial cells demon- g transgene expression for over 5 weeks. In another study, cul- endothelial cells from a Yucatan minipig were transduced *in* with replication-defective retrovirus prior to reintroduction into ry by means of a special double-balloon catheter. By occlud- ood flow to a denuded segment of the artery, the catheter pro- a temporary protected space where the modified endothelial could reattach to the vessel wall (Nabel *et al.*, 1989).

o Strategies. *In vivo* gene delivery obviates the need for cic cells and will be required for therapeutic applications such he treatment of atherosclerosis. *In vivo* gene transfer has been ed using the double-balloon catheter approach with instillation DNA delivery system into the protected space of the tempo- occluded vessel. Retroviruses, liposomes, and adenoviral vec- all have been used to target a specific site within a large vessel this approach.

sclerosis. A variety of genes have been expressed by *in vivo* ransfer for the purpose of developing useful clinical applica- s as well as for developing models of pathogenic mechanisms. cular cell proliferation and extracellular matrix protein deposition sociated with atherosclerotic narrowing of arteries. Factors that tially contribute to this process can be studied by overexpress- their genes in arterial segments. For example, when acidic fi- blast growth factor (FGF-1) is ectopically expressed in porcine s, the vessel wall becomes thickened (intimal hyperplasia) as lt of smooth muscle cell proliferation (Nabel *et al.*, 1993c). In tion, new blood vessels form within the arterial wall as a result ndothelial cell migration and growth. In contrast, when TGF-β1 ressed ectopically in the vessel, extracellular matrix synthesis timal thickening result (Nabel *et al.*, 1993a). Platelet-derived th factor B also has been shown to induce intimal hyperplasia lowing *in vivo* gene transfer (Nabel *et al.*, 1993b). These experi- tally induced changes in the vessel wall mimic the changes found herosclerotic lesions. Gene transfer thus provides a useful tool udy the effects of agents that may be part of a complex disease cess.

lammune Vasculitis. In an attempt to model another arterial dis- autoimmune vasculitis, a foreign histocompatibility gene was ered to vessel walls by liposome-mediated gene transfer, result- in a focal immune response at the site of gene transfer that his- ically resembles Takayasu arteritis (Nabel *et al.*, 1992). These riments demonstrate that models of human disease can be de- ed by introducing specific molecular changes in the blood ves- sel. These models of arterial disease may be useful in evaluating agents that can block these processes and alter the progression of the disease.

Prevention of Restenosis. In addition to understanding the process by which vascular diseases develop, gene transfer techniques have been developed to treat these diseases. For example, atherosclerotic coronary arteries often can be treated by balloon angioplasty. The narrowed segment of the atherosclerotic vessel is mechanically dilated by the insertion and inflation of a balloon catheter. Although it provides long-term benefits for many patients, this procedure has a high rate of vessel closure (restenosis) within weeks after the dilation. Restenosis occurs, in part, as a result of smooth muscle hyperplasia. Introduction of an adenoviral vector encoding thymidine kinase followed by systemic administration of ganciclovir blocked arterial hyperplasia in an animal model of restenosis (Ohno *et al.*, 1994).

Cancer Gene Therapy

Cancer gene therapies have employed several strategies that rely on unique molecular targets found in cancer cells. Activated oncogenes or mutated tumor suppressor genes are common features of human malignancies. For instance, mutations in the Kirsten-*ras* oncogene, which occur commonly in adenocarcinomas of the lung, are associated with tobacco consumption and may contribute to tumor progression. Mutations in tumor suppressor genes also occur frequently in human cancers. The retinoblastoma p53 gene, which encodes the nuclear protein p53 that regulates cell growth, is the most frequently altered gene in cancer; defects in the function of this suppressor gene and its gene product contribute to unregulated cellular proliferation.

Molecular processes that regulate cell growth, although fundamental to tumor progression, are in general difficult to target with current gene transfer methods for several reasons. Particular oncogenes, such as Kirsten-*ras*, are commonly but not uniformly present in all tumors, even of a given histological type. More important, interruption of a specific oncogene's function or restoration of tumor suppressor gene function would have to be done in every malignant cell, since untreated cells would readily divide. Because most cancers exert their morbidity and mortality through metastatic spread, one is faced with not only targeting every cancer cell but also targeting cancer cells in widespread anatomical locations (bone, liver, lung, brain, *etc.*). Furthermore, many lesions are microscopic metastatic deposits, undetectable by current diagnostic imaging methods. This makes it difficult to assess the efficacy of a new gene transfer method because, in the course of the long follow-up required, it may be unclear whether failure of the treatment resulted from inefficient gene transfer or from any of the many other events that could contribute to ineffectiveness of cancer therapy.

Many tumors acquire a series of genetic defects as they progress. In addition, some tumors arise as a consequence of mutations that result in a gain of function, not loss of function, and thus require ablation of the new activity. For example, chronic myelogenous leukemia occurs as the result f expression of a new chimeric gene product.

Because current gene transfer techniques are unable to achieve a satisfactorily high level of transfer efficiency in an *in vivo* setting, alternative strategies that do not require 100% efficiency of gene transfer have been sought. Two general approaches have evolved that may be effective when only a minority of the tumor cells are transduced: (1) cell-targeted suicide, achieved by directing the synthesis of a toxic metabolite that can permeate the tumor microenvironment, and (2) engineering an immune response t the tumor cells by ectopic cytokine expression or other means for immune recognition or activation.

Cell-targeted Suicide. Conversion of a prodrug to a t xic metabolite by genetically engineering the tumor cell is an attractive way to create an "artificial" difference between normal and neoplastic tissue. This can be achieved by the expression of a gene that confers a dominant, negatively selectable phenotype to the cancer cell, such as cell death imparted by expression of a drug-metabolizing enzyme. A variety of enzymes are capable of performing such a function, and typically kill cells by activation of a relatively nontoxic prodrug to a cytotoxic form (Table 5–2). Greater selectivity in killing malignant cells will be obtained if the transferred gene is not normally found in human beings (*e.g.*, HSV–thymidine kinase), rather than by overexpressing an endogenous gene (*e.g.*, deoxycytidine kinase).

Insertion of the HSV–thymidine kinase (HSV-TK) gene into malignant cells in conjunction with the systemic administration of ganciclovir has become a prototypic gene therapy system that uses the enzyme-prodrug approach. Many investigators have shown that the expression of the HSV-TK gene confers a negative selectable phenotype to cancer cells both *in vitro* and *in vivo*.

Moolten (1986) demonstrated acquired ganciclovir sensitivity in a murine sarcoma cell line transduced with a retroviral vector that produces HSV-TK. The transduced sarcoma tumor cells were 200 to 1000 times more sensitive to ganciclovir than control tumor cells. This finding has been reproduced in several rodent and human cancer model systems including lung cancer, mesothelioma, hepatocellular carcinoma, leukemia, melanoma, and CNS tumor models. The efficacy of this approach varies significantly and may be due to a variety of factors including promoter function, target cells studied, and efficiency of transduction.

The tumoricidal activity of the HSV-TK/ganciclovir system is due to several factors. In dividing cells, the phosphorylated ganciclovir inhibits DNA synthesis. This effect is not confined to cells that are directly transduced with HSV-TK, as neighboring cells are also affected. This phenomenon, which likely occurs as a result of several mechanisms, has been termed the "bystander effect" and has been observed in several tumor types, including CNS tumors (Freeman *et al.*, 1993). Transfer of the phosphorylated ganciclovir between cells ("metabolic cooperation") via gap junctions has been proposed as a possible mechanism. Phagocytosis by neighboring cells of ganciclovir phosphate–containing apoptotic vesicles (from dying transduced cells) also has been proposed. Immune-mediated processes also may account for significant killing of non-transduced cells. In one report, anti-tumor immunity was observed following TK-mediated killing of experimental brain tumors. Whether the tumor immunity is TK dependent, or merely a manifestation of inherent tumor cell immunogenicity, has yet to be established in this rodent model (Barba *et al.*, 1994).

More recently, adenovirus vectors have been used for gene transfer of HSV-TK. Chen *et al.* (1994a) demonstrated regression of experimental gliomas following *in vivo* adenovirus-mediated gene transfer and ganciclovir treatment. The tumor deposits were not completely eliminated by this treatment, however. Tumor cells close to the injection site were more readily transduced than were those distant, as judged by parallel marker gene transfer experiments. Furthermore, these more distant cells escaped ganciclovir toxicity because of a diminished bystander effect attributed to a paucity of gap junctions in the rodent brain tumor cell line employed. This limitation potentially can be overcome in the clinical setting by more precise stereotactic treatment planning (aided by MRI and PET studies) and by multiple tumor injections.

Other approaches have focused on introducing genes that stimulate an immune response to the tumor. Although some have argued that tumor growth occurs as a result of im-

Table 5–2
Enzyme-Prodrug Combinations for Cancer Gene Therapy

GENE	PRODRUG
HSV thymidine kinase (HSV-TK)	Ganciclovir Acyclovir
VSV thymidine kinase	Ara-M
Deoxycytidine kinase	Ara-C Fludarabine 2-Chlorodeoxyadenosine Difluorodeoxycytidine
Cytosine deaminase	5-Fluorocytidine
Nucleoside phosphorylase*	MeP-dR

*Nucleoside phosphorylase is encoded by the *E. coli DeoD* gene, the coding sequence used in this therapeutic strategy.

Key: HSV, herpes simplex virus; VSV, vesicular stomatitis virus; Ara-C, cytosine arabinoside or cytarabine; Ara-M, 6-methoxypurine arabinoside; MeP-dR, 6-methylpurine-2′-deoxyriboside.

une stimulation, there is little direct evidence t support hypothesis in most human tumors. Rather, there is a ving body of evidence that suggests that tumor cells press unique determinants that are capable of being rec-nized by the immune system.

topic Cyt kine Expressions. A variety of cytokines ve been shown to decrease tumor growth when ectopi- expressed on tumor cells or in their microenviron- (Tepper and Mule, 1994). Tumor cells engineered to rete certain cytokines have been observed to be less t form tumors when implanted in syngeneic hosts, eas their *in vitro* growth is unaffected, suggesting host factors are induced in response to the cytokines t decrease tumorigenicity. Some immunostimulatory s do not alter the growth rate of the tumor initially, ead t immunity against tumor growth if the animal later challenged with wild-type tumor cells. It is ap- nt that genetically engineered tumor cells elicit a y f host immune responses depending on the im- nomodulatory agent employed. For example, inter- kin-4 (IL-4) secretion by a tumor cell elicits a potent inflammatory response without any effect on distant r cells or tumor cells administered at later times. In trast, granulocyte-macrophage colony stimulating fac- GM-CSF) has little effect on the tumorigenicity, but s a potent anti-tumor immunity (Dranoff *et al.*, 1993). nany instances, multiple immune effects are initiated umors expressing immunomodulatory agents. This is n tumors secreting interleukin-2, where the tumor be- s infiltrated with T lymphocytes, activated macro- ges, natural killer cells, neutrophils, and eosinophils. lonally, a cytokine may have different effects in dif- tumor types. For example, interleukin-6 can have ct antiproliferative effects, recruit natural killer cells, rve as an autocrine growth factor, depending on the of tumor investigated. In many circumstances, it is ult to distinguish the effects that are induced by the kine from the effects mediated secondarily by the other ne effector cells. This has led to a rather empiric ap- h to cytokine-based cancer gene therapy. The cyto- s interleukin-1, -2, -4, -6, -7, and -12, tumor necrosis r-α (TNF-α), interferon gamma, G-CSF, GM-CSF, ymphocyte co-stimulatory molecules, all have been n to induce immune destruction of tumor cells in el systems. Of these, interleukin-2, interleukin-4, α, interferon gamma, and GM-CSF have been entered linical trials using tumor cells genetically engineered ecrete the cytokine (Tepper and Mule, 1994; *see also* ter 52).

Immune Enhancement. Other approaches aimed at increasing the immune response to cancer cells have been developed. One such approach is t express highly immunogenic molecules on the surface of cancer cells, such as expression f allotypic MHC antigens. Alternatively, rather than express an exogenous "rejection" antigen, tumor cells may be modified so that the endogenous weakly immunogenic tumor-associated antigens are better recognized. It has been long known that additional "co-stimulatory" pathways distinct from the T-cell receptor are needed to achieve T-cell activation (*see* Chapter 52). The molecules B7-1 and B7-2 stimulate one such pathway. The B7's, whose expression normally is limited to antigen-presenting cells and other specialized immune effector cells, engage specific receptors (CD-28 and CTLA-4) on the T-cell surface in concert with antigen binding to the T-cell receptor. Subsequently, T-cell activation, cell proliferation, and cytokine production ensue, and can lead to the elaboration of antitumor immunity. The absence of a costimulatory signal at the time of T-cell receptor engagement is not a neutral event; rather, it results in the development of tumor-specific anergy, not mere failure to activate the T cell (*see* Chapter 52). Thus, the simple presence of antigens in tumor cells would be expected to produce an immune-tolerant state rather than an immune-responsive state if costimulatory events do not take place. In effect, this is what is seen in most clinical situations where human tumors grow apparently unimpeded by host immune mechanisms. When some tumor cells are provided with co-stimulatory molecules, effective T-cell activation takes place. This has been demonstrated by ectopic expression of B7 on tumor cells, which then are used to stimulate an immune response to the parental tumor cell line.

Several investigators have employed this experimental approach to demonstrate that tumors endowed with B7 co-stimulation ability are able to activate the host immune system to recognize and eradicate tumor cells. Chen *et al.* (1994b) coexpressed B7 and the human papilloma virus E7 rejection antigen in K1735 murine melanoma cells. When injected into syngeneic mice, these cells (E7+B7+) induced a B7-dependent immune response, which resulted in tumor regression. In contrast, E7+B7− tumor cells did not induce an antitumor response. Furthermore, once primed by E7+B7+ cells, mice were capable of rejecting subsequently injected E7+B7− tumor cells. However, these mice were not able to reject the parental tumors, which were E7−. This study also revealed that immune rejection required the presence of CD8+ but not CD4+ T cells.

A similar study by Li *et al.* (1994) suggested the contribution of both CD8+ and CD4+ cells in tumor immunity. A K1735 cell line expressing both MHC class I and II molecules was transfected to express both B7-1 and p97 antigen. The p97 antigen is known to be very immunogenic and to stimulate the production of CD4+ clones specific for this antigen. B7 expression, when coexpressed

with p97, supported the expansion of both the CD8+ cytotoxic T lymphocytes and CD4+ lymphocytes. Furthermore, while CD8+ T cells were the most important effector cells, both cell types were necessary to eliminate established tumor nodules. Clinical experience clearly demonstrates that the mere presence of tumor-associated antigens does not induce an immune response. The implication of these studies is that the ineffectiveness of tumor antigens may be overcome by expressing B7 on the tumor cells. In these and other experiments, the presence of MHC class II molecules on the tumor cell surface, in addition to class I molecules, contributes to the overall immune response, and in particular the CD4+ component of the response. Because most human tumors do not express class II molecules, an effective CD4+ T-cell response may require additional intervention beyond B7 expression. Consequently, providing cytokine stimulation that can provide this effect may be of merit.

The foregoing experiments were performed with what is now known as B7-1. Additional experiments have shown that other molecules (B7-2, and perhaps others) are able to bind the same T-cell receptors as B7-1 (CD-28 and CTLA-4) and activate T-cell co-stimulatory pathways. The differential role of these similar ligands is only beginning to be explored. The temporal course and relative level of their expression are clearly different, as is their ability to be differentially regulated by the same stimuli. A similar level of complexity is emerging for the B7 receptors CTLA-4 and CD-28. Although the differential role of these molecules as they relate to the normal function of the immune system is beginning to be understood, which of the B7's will provide the most effective route to antitumor immunity is unknown (*see* Chapter 52 for a review of cellular mechanisms of immune enhancement and suppression).

T-cell activation, although critically dependent on TCR and co-stimulation pathways, also may be supported by additional functions normally provided by the antigen-presenting cell. Interleukin-12 (IL-12) is secreted by antigen-presenting cells and functions by binding to specific receptors on T cells and natural killer cells. IL-12 induces the production of interferon gamma and enhances the production of a cytotoxic T-lymphocyte response. In one murine tumor model, IL-12, when produced in the microenvironment of a developing tumor nodule, delayed development of detectable tumor nodules (Ohno *et al.*, 1994). IL-12 in this model did not lead to protective antitumor immunity, *i.e.*, tumor development was delayed, but not entirely prevented. Interestingly, the B16-derived BL-6 melanoma cell line is poorly immunogenic yet was able to provoke T-cell activation when supported by this exogenous cytokine. Other investigators have reported that B16 tumor cell lines are not rendered capable of inducing an immune response when transduced to express B7-1. The fact that IL-12 can induce immune responsiveness to a tumor when B7-1 could not suggests that these immunomodulatory molecules may provide different functions. Recently, it has been shown that B7-1 and IL-12 can act in synergy to induce T-cell proliferation and cytokine (interferon gamma and TNF-α) production (see Chapter 52).

Not all of the obstacles to genetically engineered tumor vaccines have been fully identified. Immune tolerance of tumor cells may arise by many mechanisms, including tumor cell secretion of immunosuppressive agents (*e.g.*, TGF-β), and other means to overcome tolerance will need to be devised. Nonetheless, the ectopic expression of genes in cancer cells is a very flexible and powerful tool that likely will improve upon the current therapeutic approach of systemically administered antineoplastic agents (*see* Chapter 51).

Gene Transfer into Hematopoietic Stem Cells

Gene transfer int bone marrow stem cells has been proposed for a variety of inherited and acquired disorders. These include inherited defects in cells produced by the bone marrow (*e.g.*, sickle cell disease, thalassemias, chronic granulomatous disease, and several lymphocyte disorders), as well as acquired illnesses in which marrow-derived cells are secondarily involved (*e.g.*, acquired immunodeficiency syndrome [AIDS] and chemotherapy-induced myelosuppression). The long-term repopulating potential of the bone marrow stem cell also makes it a potentially useful agent for the production and delivery of proteins normally produced by nonhematopoietic cells (*e.g.*, coagulation proteins). The development of bone marrow transplantation has provided substantial precedence for this approach. The growing number of diseases that can be treated effectively by bone marrow transplantation demonstrates the therapeutic efficacy of providing a "corrected" marrow. For example, severe β-thalassemia (an inherited defect in hemoglobin biosynthesis) can be cured by transplantation of bone marrow from a normal donor. The gene therapy equivalent would be to correct the patients' own marrow rather than substitute a "foreign" normal marrow. Because bone marrow can be removed easily and reimplanted, it provides an ideal setting for *ex vivo* gene therapy strategies. The ultimate goal is to be able to transfer genes into hematopoietic stem cells and allow these cells to reconstitute the bone marrow with the selective expression of the transferred gene in a specific hematopoietic cell lineage.

Immunodeficiency Disorders. Gene therapy offers potential treatments for a variety of immunodeficiency disorders. As noted earlier, the first disorder to be treated by gene therapy was a form of severe combined immunodeficiency (SCID) caused by the deficiency of the enzyme adenosine deaminase (ADA). In children with this disorder, the absence of ADA leads to an accumulation of deoxyadenosine triphosphate, which is toxic to lymphocytes; patients develop recurrent life-threatening infections due to defective cell-mediated and humoral immune responses. Current standard therapy includes bone marrow transplantation from an HLA-matched sibling. Although less effective, intravenous replacement of ADA is used in patients who lack a suitable marrow donor. While the first clinical trial of gene therapy for ADA deficiency resulted in clinical improvement, it has not provided a permanent cure. The first patients were treated by repeated gene transfer

peripheral blood lymphocytes that had been isolated pheresis. A preferable approach would be to insert the DA gene into pluripotent hematopoietic stem cells that uld reconstitute the immune system with a complete rtoire of immune cells. Such approaches are under depment. It has been dem nstrated recently that longm correction of ADA deficiency can be achieved (albeit w levels) in a rhesus monkey model (Van Beusechem ., 1992; Bodine *et al.*, 1993).

Leukocyte adhesion deficiency (LAD) is another inited disorder that results from defective leukocyte func- Patients with this disorder lack cell surface glycoeins that mediate cell-cell interactions necessary for mune function. Krauss *et al.* (1991) have developed a virus-mediated gene therapy strategy for the treatment ese disorders.

sosomal Storage Diseases. Lysosomal storage dis- result from the lysosomal accumulation of cellular erial that cannot be degraded, or degraded material that not be further processed. Over fifty such disorders are n in human beings and animals. In these disorders, bsence f a particular lysosomal enzyme involved in breakdown of glycolipids and sphingolipids leads to an ease in lysosome size and number, and secondary deement f cellular function. The recessively inherited cher disease is typical of the storage diseases in many ects. Glucosylceramide, a lipid, accumulates in ophages f affected individuals due to a deficiency of ocerebrosidase. This results in enlargement of the liver l spleen, destructive bone lesions, and variable central ous system dysfunction. Several genetic defects are n and there is significant variation in the phenotypic earance f the disease within a given genotype (*see* ufeld *et al.*, 1991).

The observation that cultured fibroblasts from an affected indiual could be "cross-corrected" by coculture with normal cells that rete the enzyme led to the development of replacement therapy. ugh intravenous administration of the deficient enzyme is not y effective in patients, replacement therapy has demonstrated enzyme-deficient cells are able to take up exogenously produced yme. Alternatively, transplantation of an affected patient with norbone marrow cells can offer clinical improvement in some cases osomal storage disease. The transplanted hematopoietic cells ble to deliver normal enzyme to affected tissues. Cells capable making the normal enzyme can transfer the secreted enzyme to a dent cell by a receptor-mediated endocytosis pathway or via dicontact-mediated transfer. This capacity for cell-to-cell transfer ysosomal enzymes via receptor-mediated endocytosis has been nstrated in a number of animal models, including a murine el of β-glucuronidase deficiency (Bou-Gharios *et al.*, 1993) and ine model of α-mannosidosis (Walkley *et al.*, 1994). Although bone marrow transplant may be therapeutically useful in some circumstances, its utility is diminished by the availability of suitable marrow donors and the immunosuppressive risks associated with transplanting allogeneic bone marrow. Gene transfer methods that may overcome these shortcomings are being developed. By engineering the patient's marrow to express the desired enzyme, the patient's own leukocytes could deliver normal enzyme. In one proposed treatment strategy, bone marrow would be harvested from the patient and the "corrected" gene inserted in *in vitro* culture. Reinfusion of the manipulated marrow cells would lead to the long-term replacement of the enzyme without the need for immunosuppressive agents. Several investigators have effected retrovirus-mediated gene transfer into marrow cells from animals and human beings and demonstrated that long-term production of the desired enzyme is achievable.

Drug Resistance Genes in the Treatment of Cancer. The mechanisms by which cancer cells are able to survive the cytotoxic effects of chemotherapy are well described for a number of chemotherapeutic agents. These mechanisms include the expression of genes that are able to inactivate or eliminate the toxic drug (*see* Chapter 51). Although these genes currently serve to limit the effectiveness of many chemotherapy regimes, it is possible that they might be redeployed to have the opposite effect, that is, to protect normal tissues from the toxic effects of chemotherapy. One gene in particular has received much attention in this regard, the multidrug resistance (MDR-1) gene encoding the multidrug transporter protein (also known as P-glycoprotein). This transmembrane protein is capable of pumping a wide variety of chemotherapeutic agents (*e.g.*, adriamycin, vinca alkaloids, epipodophyllotoxins, and taxol) and other drugs out of cells, thus protecting them from the agents' toxic effects (Gottesman *et al.*, 1994). Many cancers display a dose-dependent sensitivity to chemotherapy, whereby larger doses of chemotherapy lead to greater tumor regression and improved survival (*see* Chapter 51). This is best illustrated by testicular cancers, which are highly curable when treated aggressively. Unfortunately, toxicity to normal tissues, especially the bone marrow, limits the use of larger doses of chemotherapy in many cancers. To overcome this, autologous bone marrow transplantation has been employed to rescue the bone marrow from the toxic effects of high-dose chemotherapy. In some cancers (*e.g.*, breast cancer and testicular cancer), relapse after standard therapy can be treated by harvesting uninvolved normal bone marrow prior to high-dose chemotherapy. The stored autologous marrow is then reinfused to rescue the patient from therapy-induced marrow ablation. Such high-dose chemotherapy with autologous bone marrow transplantation is now standard therapy for relapsed testicular cancer. Capitalizing on this concept, a gene therapy-based strategy has been proposed whereby

the MDR-1 gene would be used to render the bone marrow resistant to the toxic effects of the chemotherapy (Gottesman *et al.*, 1994).

Although gene transfer into marrow stem cells leads to transgene expression in only a few percent of hematopoietic cells, successive cycles of chemotherapy can be used to enrich for transduced marrow cells. This approach may be applied to cancers that demonstrate a steep dose-response t chemotherapy and where myelosuppression is the dose-limiting toxicity.

Gene Therapy for Infectious Diseases.

The failure of conventional antibiotics to treat many types of serious pathogenic agents effectively, most notably the human immunodeficiency virus, and the availability of unique molecular targets in these pathogens have encouraged the exploration of gene therapies for infectious diseases.

AIDS. Nabel *et al.* (1994) and Malim *et al.* (1992) have used a dominant negative mutant protein in designing a gene transfer strategy for the treatment of AIDS. The rev protein, produced by the human immunodeficiency virus, is a regulatory protein necessary for viral replication. It binds to a specific viral RNA motif (rev response element, RRE) and promotes the synthesis of new viral proteins. Studies in experimental models have shown that by introducing a mutant *rev* gene, the HIV-infected cell produces an altered rev protein. This protein, called Rev M10, is capable of binding the same motif as the normal rev, but is not functional in promoting the synthesis of new viral proteins. Consequently, Rev M10 competitively inhibits the activity of the normal rev protein and ultimately attenuates HIV replication.

Immunization. By an entirely different approach, gene transfer can be employed to drive the synthesis of an antibody with predetermined specificity. This would eliminate the need to rely on a variable or unpredictable immun response t a vaccine (particularly in immune-compromised patients) and could be used t direct the synthesis f the antibody to a specific site. Chen *et al.* (1994b) recently have described a single-chain antibody with specificity for the gp120 HIV protein that can be delivered by gene transfer. They have shown that human CD4+ T lymphocytes can be transduced to express this antibody intracellularly, and that cytopathic syncytium formation and HIV-1 production were inhibited, although not eliminated.

PROSPECTUS

Human gene therapy, although still in the infant stages of development, offers the possibility for major advances in the prevention and treatment of myriad diseases. Gene therapy brings an entirely new paradigm for the treatment of disorders stemming from missing or defective genes, whether they are inherited or acquired. Furthermore, this technology likely also will evolve for the treatment of "nongenetic" illnesses, where the tissue-specific synthesis of a protein can be used for therapeutic benefit. The identification of new genes related to specific diseases will broaden the scope of applications. Currently, however, the clinical application of gene therapy is more limited by the availablity of suitable gene transfer methodology than by the identification of suitable targets for genetic alteration. However, as increasing numbers of investigators address these issues, better reagents likely will emerge. Furthermore, a better understanding of the pathophysiological processes will permit the design of physiologically appropriate interventions. It is to be hoped that increased collaboration among physicians, molecular biologists, and cell biologists will result in the development of highly integrated approaches to this new form of therapy.

BIBLIOGRAPHY

Anderson, W.F., Blaese, R.M., and Culver, K. The ADA human gene therapy protocol. *Hum. Gene Ther.*, 1990, *1*:331–341.

Armentano, D., Soukdeo, C., White, G., Giuggio, V., Souza, D., Couture, L., Cardona, L., Vincent, K., Wadsworth, S., and Smith, A. Second generation adenovirus vectors for cystic fibrosis gene therapy. *J. Cell Biochem. Suppl.*, 1994, *18A*:222.

Barba, D., Hardin, J., Sadelain, M., and Gage, F.H. Development of anti-tumor immunity following thymidine kinase-mediated killing of experimental brain tumors. *Proc. Natl. Acad. Sci. U.S.A.*, 1994, *91*:4348–4352.

Batra, R.K., Wang-Johanning, F., Wagner, E., Garver, R.I., and Curiel, D.T. Receptor-mediated gene delivery employing lectin-binding specificity. *Gene Therapy*, 1994, *1*:255–260.

Bett, A.J., Haddara, W., Prevec, L., and Graham, F.L. An efficient and flexible system for construction of adenovirus vectors with insertions or deletions in early regions 1 and 3. *Proc. Natl. Acad. Sci. U.S.A.*, 1994, *91*:8802–8806.

Bodine, D.M., Moritz, T., Donahue, R.E., Luskey, B.D., Kessler, S.W., Martin, D.I.K., Orkin, S.H., Nienhuis, A.W., and Williams, D.A. Long-

...term *in vivo* expression of a murine adenosine deaminase gene in Rhesus monkey hematopoietic cells of multiple lineages after retroviral mediated gene transfer into CD34⁺ bone marrow cells. *Blood*, 1993, 82:1975–1980.

...-Charlos, G., Adams, G., Pace, P., Warden, P., and Olsen, L. Correction of a lysosomal deficiency by contact-mediated enzyme transfer after bone marrow transplantation. *Transplantation*, 1993, 56:991–996.

...oviatzis, E.J., Chase, M., Wei, M.X., Tamiya, T., Hurford, R.K., Jr., Kowall, N.W., Tepper, R.I., Breakefield, X.O., and Chiocca, E.A. Gene transfer into experimental brain tumors mediated by adenovirus, herpes simplex virus, and retrovirus vectors. *Hum. Gene Ther.*, 1994, 5:183–191.

...igham, K., Meyrick, B., Christman, B., Magnuson, M., King, G., and Berry, L., *In vivo* transfection of murine lungs with a functioning prokaryotic gene using a liposome vehicle. *Am. J. Med. Sci.*, 1989, 298:278–281.

...sonico, A.E., Conary, J.T., Meyrick, B.O., and Brigham, K.L. Aerosol and intravenous transfection of human α 1-antitrypsin gene to lungs of rabbits. *Am. J. Resp. Cell. Mol. Biol.*, 1994, 10:24–29.

...hen, S.-H., Shine, H.D., Goodman, J.C., Grossman, R.G., and Woo, S.L. Gene therapy for brain tumors: regression of experimental gliomas by adenovirus-mediated gene transfer *in vivo*. *Proc. Natl. Acad. Sci. U.S.A.*, 1994a, 91:3054–3057.

...hen, S.-Y., Bagley, J., and Marasco, W.A. Intracellular antibodies as a new class of therapeutic molecules for gene therapy. *Hum. Gene Ther.*, 1994b, 5:595–601.

...owdhury, J.R., Grossman, M., Gupta, S., Chowdhury, N.R., Baker, J.R., Jr., and Wilson, J.M. Long-term improvement of hypercholesterolemia after *ex vivo* gene therapy in LDLR-deficient rabbits. *Science*, 1991, 254:1802–1805.

...ary, J.T., Parker, R.E., Christman, B.W., Faulks, R.D., King, G.A., Meyrick, B.O., and Brigham, K.L. Protection of rabbit lungs from endotoxin injury by *in vivo* hyperexpression of the prostglandin G/H synthase gene. *J. Clin. Invest.*, 1994, 93:1834–1840.

...tiano, R.J., Smith, L.C., Kay, M.A., Brinkley, B.R., and Woo, S.L.C. Hepatic gene therapy: efficient gene delivery and expression in primary hepatocytes utilizing a conjugated adenovirus-DNA complex. *Proc. Natl. Acad. Sci. USA*, 1993, 90:11548–11552.

...ver, K.W., Anderson, W.F., and Blaese, R.M. Lymphocyte gene therapy. *Hum. Gene Ther.*, 1991, 2:107–109.

...avis, H.L., Demeneix, B.A., Quantin, B., Coulombe, J., and Whalen, R.G. Plasmid DNA is superior to viral vectors for direct gene transfer into adult mouse skeletal muscle. *Hum. Gene Ther.*, 1993, 4:733–740.

...nnoff, G., Jaffee, E., Lazenby, A., Golumbek, P., Levitsky, H., Brose, K., Jackson, V., Hamada, H., Pardoll, D., and Mulligan, R.C. Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. *Proc. Natl. Acad. Sci. U.S.A.*, 1993, 90:3539–3543.

...Engelhardt, J.F., Litzky, L., and Wilson, J.M. Prolonged transgene expression in cotton rat lung with recombinant adenovirus defective in E2a. *Human Gene Therapy*, 1994, 5:1217–1229.

Felgner, P., Gadek, T., Holm, M., Roman, R., Chan, H.W., Wenz, M., Northrop, J.P., Ringold, G.M., and Danielsen, M. Lipofection: a highly efficient, lipid-mediated DNA transfection procedure. *Proc. Natl. Acad. Sci. U.S.A.*, 1987, 84:7413–7417.

Fisher, K.J., and Wilson, J.M. Biochemical and functional analysis of an adenovirus-based ligand complex for gene transfer. *Biochem. J.*, 1994, 299:49–58.

...eeman, S.M., Abboud, C.N., Whartenby, K.A., Packman, C.H., Koeplin, D.S., Moolten, F.L., and Abraham, G.N. The "bystander effect": tumor regression when a fraction of the tumor mass is genetically modified. *Cancer Res.*, 1993, 53:5274–5283.

Fynan, E.F., Webster, R.G., Fuller, D.H., Haynes, J.R., Santoro, J.C., and Robinson, H.L. DNA vaccines: protective immunizations by parenteral, mucosal, and gene-gun inoculations. *Proc. Natl. Acad. Sci. U.S.A.*, 1993, 90:11478–11482.

Gottesman, M.M., Germann, U.A., Akseltijevich, L., Sugimoto, Y., Cardarelli, C.O., and Pastan, I. Gene transfer of drug resistance genes: implications for cancer therapy. *Ann. N.Y. Acad. Sci.*, 1994, 716:126–138.

Graham, F.L., and Prevek, L. Manipulation of adenoviral vectors. In, *Methods in Molecular Biology: Gene Transfer and Expression Protocols*. The Humana Press, Inc., Clifton, NJ, 1991, pp. 109–128.

Greber, U.F., Willetts, M., Webster, P., and Helenius, A. Stepwise dismantling of adenovirus 2 during entry into cells. *Cell*, 1993, 75:477–486.

Grossman, M., Raper, S.E., Kozarsky, K., Stein, E.A., Engelhardt, J.F., Muller, D., Lupien, P.J., and Wilson, J.M. Successful *ex vivo* gene therapy directed to liver in a patient with familial hypercholesterolaemia. *Nat. Genet.*, 1994, 6:335–341.

Horwitz, M.S. Adenoviruses. In *Virology*, 2nd ed. Raven Press, New York, 1990, pp. 1723–1740.

Hyde, S.C., Gill, D.R., Higgins, C.F., and Trezise, A.E.O. Correction of the ion transport defect in cystic fibrosis transgenic mice by gene therapy. *Nature*, 1993, 362:250–255.

Jolly, D. Viral vector systems for gene therapy. *Cancer Gene Therapy*, 1994, 1:51–64.

Kennedy, P.G.E., and Steiner, I. The use of herpes simplex virus vectors for gene therapy in neurological diseases. *Q. J. Med.*, 1993, 86:697–702.

Kotin, R.M. Prospects for the use of adeno-associated virus as a vector for human gene therapy. *Hum. Gene Ther.*, 1994, 5:793–801.

Kozarsky, K.F., McKinley, D.R., Austin, L.L., Raper, S.E., Stratford-Perricaudet, L.D., and Wilson, J.M. *In vivo* correction of low density lipoprotein receptor deficiency in the Watanabe heritable hyperlipidemic rabbit with recombinant adenoviruses. *J. Biol. Chem.*, 1994, 269:13695–13702.

Krauss, J.C., Bond, L.M., Todd, R.F.I., and Wilson, J.M. Expression of retroviral transduced human CD18 in murine cells: an *in vitro* model of gene therapy for leukocyte adhesion deficiency. *Hum. Gene Ther.*, 1991, 2:221–228.

Ledley, T.S., and Ledley, F.D. Multicompartment, numerical model of cellular events in the pharmacokinetics of gene therapies. *Hum. Gene Ther.*, 1994, 5:679–691.

Li, Y., McGowan, P., Hellstrom, I., Hellstrom, K.E., and Chen, L. Costimulation of tumor-reactive CD4+ and CD8+ T lymphocytes by B7, a natural ligand for CD28, can be used to treat established mouse melanoma. *J. Immunol.*, 1994, 153:421–428.

Lotze, M.T. Transplantation and adoptive cellular therapy of cancer: the role of T-cell growth-factors. *Cell Transplant.*, 1993, 2:33–47.

Lotze, M.T., and Rubin, J.T. Gene therapy of cancer: a pilot study of IL-4-gene-modified fibroblasts admixed with autologous tumor to elicit an immune response. *Hum. Gene Ther.*, 1994, 5:41–55.

Lotze, M.T., and Rubin, J.T. The treatment of patients with melanoma using interleukin-2, interleukin-4 and tumor infiltrating lymphocytes. *Hum. Gene Ther.*, 1992, 3:167–177.

Malim, M.H., Freimuth, W.W., Liu, J., Boyle, T.J., Lyerly, H.K., Cullen, B.R., and Nabel, G.J. Stable expression of transdominant Rev protein in human T cells inhibits human immunodeficiency virus replication. *J. Exp. Med.*, 1992, 176:1197–1201.

Michael, S.I., and Curiel, D.T. Strategies to achieve targeted gene delivery via the receptor-mediated endocytosis pathway. *Gene Therapy*, 1994, 1:223–232.

Miller, A.D., Miller, D.G., Garcia, J.V., and Lynch, C.M. Use of retroviral vectors for gene transfer and expression. *Methods Enzymol.*, 1993, *217*:581–599.

Moolten, F.L. Tumor chemosensitivity conferred by inserted herpes thymidine kinase genes: paradigm for a prospective cancer control strategy. *Cancer Res.*, 1986, *46*:5276–5281.

Moss, B. Poxviridae and their replication. In, *Virology*. Raven Press, New York, 1990, pp. 2079–2111.

Moss, B., and Flexner, C. Vaccinia virus expression vectors. *Annu. Rev. Immunol.*, 1987, *5*:305–324.

Nabel, E.G., Plautz, G., Boyce, F.M., Stanley, J.C., and Nabel, G.J. Recombinant gene expression *in vivo* within endothelial cells of the arterial wall. *Science*, 1989, *244*:1342–1344.

Nabel, E.G., Plautz, G., and Nabel, G.J. Transduction of a foreign histocompatibility gene into the arterial wall induces vasculitis. *Proc. Natl. Acad. Sci., U.S.A.*, 1992, *89*:5157–5161.

Nabel, E.G., Plautz, G.E., and Nabel, G.J. Recombinant growth factor gene expression in vascular cells *in vivo*. *Ann. N.Y. Acad. Sci.*, 1994a, *714*:247–252.

Nabel, E.G., Shum, L., Pompili, V.J., Yang, Z.Y., San, H., Shu, H.B., Liptay, S., Gold, L., Gordon, D., Derynck, R., and Nabel, G.J. Direct transfer of transforming growth factor beta 1 gene into arteries stimulates fibrocellular hyperplasia. *Proc. Natl. Acad. Sci. U.S.A.*, 1993a, *90*:107759–107763.

Nabel, E.G., Yang, Z., Liptay, S., San, H., Gordon, D., Haudenschild, C.C., and Nabel, G.J. Recombinant platelet-derived growth factor B gene expression in porcine arteries induces intimal hyperplasia *in vivo*. *J. Clin. Invest.*, 1993b, *91*:1822–1829.

Nabel, E.G., Yang, Z.Y., Plautz, G., Forough, R., Zhan, X., Haudenschild, C.C., Maciag, T., and Nabel, G.J. Recombinant fibroblast growth factor-1 promotes intimal hyperplasia and angiogenesis in arteries *in vivo*. *Nature*, 1993c, *362*:844–846.

Nabel, G.J., Fox, B.A., Felgner, P., Shu, S., and Cho, K. Immunotherapy for cancer by direct gene transfer into tumors. *Hum. Gene Ther.*, 1994b, *5*:57–77.

Nabel, G.J., Fox, B.A., Post, L., Thompson, C.B., and Wolfendin, C. A molecular genetic intervention for AIDS—effects of a transdominant negative form of Rev. *Hum. Gene Ther.*, 1994, *5*:79–92.

Neel, J.V. Germ-line gene therapy: another view. *Hum. Gene Ther.*, 1993, *4*:127–128.

Neufeld, E.F. Lysosomal storage diseases. *Annu. Rev. Biochem.*, 1991, *60*:257–280.

Nicolau, C., Le Pape, A., Soriano, P., Fargette, F., and Juhel, M-F. *In vivo* expression of rat insulin after intravenous administration of the liposome-entrapped gene for rat insulin I. *Proc. Natl. Acad. Sci. U.S.A.*, 1983, *80*:1068–1072.

Nienhuis, A.W., McDonagh, K.T., and Bodine, D.M. Gene transfer into hematopoietic stem cells. *Cancer*, 1991, *67*:2700–2704.

Ohno, T., Gordon, D., San, H., Pompili, V.J., Imperiale, M.J., Nabel, G.J., and Nabel, E.G. Gene therapy for vascular smooth muscle cell proliferation after arterial injury. *Science*, 1994, *265*:781–784.

Oldfield, E.H., Ram, Z., Culver, K.W., Blaese, R.M., DeVroom, H.L., and Anderson, W.F. Gene therapy for the treatment of brain tumors using intra-tumoral transduction with the thymidine kinase gene and intravenous ganciclovir. *Hum. Gene Ther.*, 1993, *4*:39–69.

Rosenberg, S.A., Aebersold, P., Cornetta, K., Kasid, A., Morgan, R.A., Moen, R., Karson, E.M., Lotze, M.T., Yang, J.C., Topalian, S.L., Merino, M.J., Culver, K., Miller, A.D., Blaese, R.M., and Anderson, W.F. Gene transfer into humans: immunotherapy of patients with advanced melanoma, using tumor-infiltrating lymphocytes modified by retroviral gene transduction. *N. Engl. J. Med.*, 1990, *323*:570–578.

Rubin, B.A., and Rorke, L.B. Adenovirus vaccines. In, *Vaccines*, 2nd ed. W.B. Saunders; Philadelphia, 1994, pp. 474–502.

Seth, P. Adenovirus-dependent release of choline from plasma membrane vesicles at an acidic pH is mediated by the penton base protein. *J. Virol.*, 1994, *68*:1204–1206.

Tatum, E.L. Molecular biology, nucleic acids and the future of medicine. *Perspect. Biol. Med.*, 1966, *10*:19–32.

Tepper, R.I., and Mule, J.J. Experimental and clinical studies of cytokine gene-modified tumor cells. *Hum. Gene Ther.*, 1994, *5*:153–164.

Valyi-Nagy, T., Gesser, R.M., Raengsakulrach, B., Deshmane, S.L., Randazzo, B.P., Dillner, A.J., and Fraser, N.W. A thymidine kinase-negative HSV-1 strain establishes a persistent infection in SCID mice that features uncontrolled peripheral replication but only marginal nervous system involvement. *Virology*, 1994, *199*:484–490.

Le van Beusechem, V.W., Kukler, A., Heidt, P.J., and Valerio, D. Long-term expression of human adenosine deaminase in rhesus monkeys transplanted with retrovirus-infected bone-marrow cells. *Proc. Natl. Acad. Sci. U.S.A.*, 1992, *89*:7640–7644.

Walkley, S.U., Thrall, M.A., Dobrenis, K., Huang, M., March, P.A., Siegel, D.A., and Wurzelmann, S. Bone marrow transplantation corrects the enzyme defect in neurons of the central nervous system in a lysosomal storage disease. *Proc. Natl. Acad. Sci. U.S.A.*, 1994, *91*:2970–2974.

Wilson, J.M., Birinyi, L.K., Salomon, R.N., Libby, P., Callow, A.D., and Mulligan, R.C. Implantation of vascular grafts lined with genetically modified endothelial cells. *Science*, 1989, *244*:1344–1346.

Wolff, J.A., and Lederberg, J. An early history of gene transfer and therapy. *Hum. Gene Ther.*, 1994, *5*:469–480.

Wolff, J.A., Malone, R.W., Williams, P., Chong, W., Acsadi, G., Jani, A., and Felgner, P.L. Direct gene transfer into mouse muscle *in vivo*. *Science*, 1990, *247*:1465–1468.

Wu, G.Y., and Wu, C.H. Receptor-mediated *in vitro* gene transformation by a soluble DNA carrier system. *J. Biol. Chem.*, 1987, *262*:4429–4432.

Yang, Y., Nunes, F.A., Berencsi, K., Furth, E.E., Gönczöl, E., and Wilson, J.M. Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy. *Proc. Natl. Acad. Sci. U.S.A.*, 1994, *91*:4407–4411.

Zabner, J., Couture, L.A., Gregory, R.J., Graham, S.M., Smith, A.E., and Welsh, M.J. Adenovirus-mediated gene transfer transiently corrects the chloride transport defect in nasal epithelia of patients with cystic fibrosis. *Cell*, 1993, *75*:207–216.

Zenke, M., Steinlein, P., Wagner, E., Cotten, M., Beug, H., and Birnstiel, M.L. Receptor-mediated endocytosis of transferrin-polycation conjugates: an effective way to introduce DNA into hematopoietic cells. *Proc. Natl. Acad. Sci. U.S.A.*, 1990, *87*:3655–3659.

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Targeted vectors for gene therapy

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ABSTRACT Successful gene therapy requires not only the identification of an appropriate therapeutic gene for treatment of the disease, but also a delivery system by which that gene can be delivered to the desired cell type both efficiently and accurately. Reductions in accuracy will inevitably also reduce efficiency since fewer particles will be available for delivery to the correct cells if many are sequestered into nontarget cells. In addition, the therapy will have net benefit to the patient only if gene delivery is sufficiently restricted such that normal cells are left unaffected by any detrimental affects of bystander cell transduction. Here we review how currently available delivery systems, both plasmid and viral, can be manipulated to improve their targeting to specific cell types. Currently, targeting is achieved by engineering of the surface components of viruses and liposomes to achieve discrimination at the level of target cell recognition and/or by incorporating transcriptional elements into plasmid or viral genomes such that the therapeutic gene is expressed only in certain target cell types. In addition, we discuss emerging vectors and suggest how gene therapy delivery systems of the future will be composites of the best features of diverse vectors already in use.—Miller, N., Vile, R. Targeted vectors for gene therapy. *FASEB J.* 9, 190–199 (1995).

Key Words: *targeting* • *retrovirus* • *adenovirus* • *liposome*

THE IDENTIFICATION OF THE UNDERLYING genetic defects has recently made gene therapy an attractive treatment option for a wide variety of diseases. However, there is a corresponding requirement to produce vector systems that can deliver therapeutic genes to the appropriate target cells either in vivo or ex vivo. These systems must be both *efficient* and *accurate*. The range of different diseases amenable to intervention by gene therapy means, however, that no single delivery system is likely to be universally appropriate. For instance, the requirements of gene therapy for cystic fibrosis are greatly different from those of cancer. In the former case, only a certain proportion of a localized population of cells needs to be targeted with a single corrective gene; by contrast, cancer gene therapy usually involves the targeting of all of a diffusely spread population of cells, with the ultimate aim of killing rather than correcting them. Hence, the stringency with which the therapeutic gene needs to be accurately delivered can vary greatly. Expression of a copy of the cystic fibrosis transporter gene in nontarget cells is likely to be much less toxic than inadvertant expression of cytotoxic genes, aimed at cancer cells, but expressed in normal bystander cells.

Here, we review the progress in targeting gene delivery systems to specific target cell populations and look forward to the areas of research that will bring developments for the future. Unfortunately, improvements in the accuracy of a vector often compromise its efficiency, and vice versa. Nonetheless, it is clear that the technology now exists to incorporate specific targeting features into most of the currently available delivery systems. These may be at the level of *1)* target cell surface recognition, by manipulating the surface recognition components of viruses and liposomes; or *2)* target cell transcriptional restrictions, by incorporating transcriptional elements into plasmid or viral genomes such that the therapeutic gene is expressed only in certain target cell types.

The ultimate aim for the vectors of the future is to include these and other targeting opportunities within the same vehicle. In all probability, this will involve the incorporation of the most beneficial features of a variety of viral and nonviral systems into a single hybrid vector specifically custom built for each individual therapeutic situation.

TARGETING OF GENE THERAPY VECTORS AT THE LEVEL OF THE CELL SURFACE

Retroviral vectors

A primary determinant of retrovirus infectivity is the interaction between specific receptors on the host cell surface and glycoproteins (Env) on the lipid envelope of the retroviral particle. Ideally, targeted retroviral vectors for human gene therapy would use safe recombinant genomes and packaging lines from wild-type retroviruses that naturally display envelope proteins with the required tropisms. However, few naturally occurring retroviral infections are strictly limited to one cell type (1), and of the known receptors for retroviruses, only the HIV-1/SIV receptor CD-4 (2) is of relatively restricted distribution. Attempts have been made to produce vectors and packaging lines from HIV (3). However, HIV is a complex retrovirus that requires a number of self-encoded autoregulatory proteins, and this complicates the construction of stable packaging lines. Nevertheless, the principle of a recombinant HIV genome as a gene vector for $CD4^+$ cells has been demonstrated (3). However, vectors carrying HIV-1 *env* sequences would have to be used with extreme caution as the HIV-Env protein itself may be neurotoxic (4) or even immunosuppressive.

Most recombinant retroviral vectors and packaging lines produced so far have been based on murine leukemia viruses (MLVs)[2] (5). There are five recognized MLV groups (1) as

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[2]Abbreviations: MLVs, murine leukemia viruses; MLV-E, ecotropic strain of MLV; MLV-A, amphotropic strain of MLV; RES, reticuloendothelial system; PEG, polyethylene glycol; ReSV, respiratory syncytial virus; ASOR, asialoorosomucoid; LCRs, locus control regions; DT-A, diphtheria toxin A; MVM, mouse minute virus.

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ined by tropism, of which the most useful for gene delivry purposes have been the ecotropic strain (MLV-E), which fects virtually all rodent cells, and the amphotropic strain LV-A), which infects practically all mammalian cells. ckaging lines hav therefore been created to allow production of retroviral vectors with host ranges that are either otropic or amphotropic, respectively (5). It is likely that all roviral vectors suitable for human gene therapy in the ar future will be based on such recombinant MLV genomes because they are well characterized with regard to fety and efficiency. For targeted retroviral vectors, then, the oblem is either to restrict the promiscuous tropism of amotropic particles or to confer upon ecotropic particles a imited human cell affinity. This could be done either by: *1)* netic manipulation of the producer line such that amphoecotropic Env is replaced by a different viral or nonviral otein having the required affinity; *2)* directly engineering particular affinity into Env; or *3)* molecular conjugate apaches, in which ligands are coupled to the outside of the roviral particle.

Replacement of Env: retroviral pseudotypes

he facility (5) with which *trans*- and *cis*-acting functions can separated in MLV packaging lines allows easy experimenal manipulation of the *trans*-acting function responsible for llular tropism, namely, Env. This raises the possibility of placing one viral *env* with that of another, thereby creating hybrid producer line that generates "pseudotyped" viral vectors with a tropism conferred by the replacement *env* (Fig. . Phenotypic mixing has been used for many years as a tool study receptor interactions (see ref 1 for a review); however, efforts have recently been directed at precisely replacing *env* and producing not envelope mixtures but vecr populations exclusively displaying a novel tropism (1, 6). uch hybrid formation in general seems to occur more efficiently between closely related viruses. For instance, a recombinant MoMLV genome can be rescued by C-type viruses but not by HTLV-I or D-type viruses (7). However, provision of homologous or more closely related Gag proteins in some cases relaxes phen typic restrictions on efficient pseudotyping of vect r genomes with exogen us Env; for instance, an MoMLV vector can be packaged inside HTLV-1 (8) envelopes when M MLV *gag-pol* are supplied in *trans*. Similarly, HIV has been given an extended host cell range by pseudotyping with the unrelated viruses HSV and VSV (9). Although these examples demonstrate the principle of creating an improved retroviral vector for human gene therapy by pseudotyping, so far they have produced only vectors with extended tropism rather than with restricted specificities.

The logical and necessary extension of pseudotyping approaches, then is to replace retroviral envelope genes with genes derived from nonviral sources. Although there are instances of nonviral glycoproteins being preferentially incorporated into retroviral particles, such as Thy-1 (10) and CD4 (11), actual infection of target cells, as opposed to specific binding, via display of such nonviral proteins has not been demonstrated, and is likely to require either fusogenic sequences within the foreign protein itself or coexpression of fusogenic molecules on the viral envelope.

Engineering Env

Genetic manipulations whereby sequences conferring specific binding affinities are engineered into preexisting viral *env* genes represent a promising approach. In MoMLV the sequences that determine receptor specificity seem to be in the most distal of the two variable regions within the amino-terminal portion of the SU Env subunit, and replacement of the variable region of one strain with that of another can, for instance, change viral tropism from that of strain

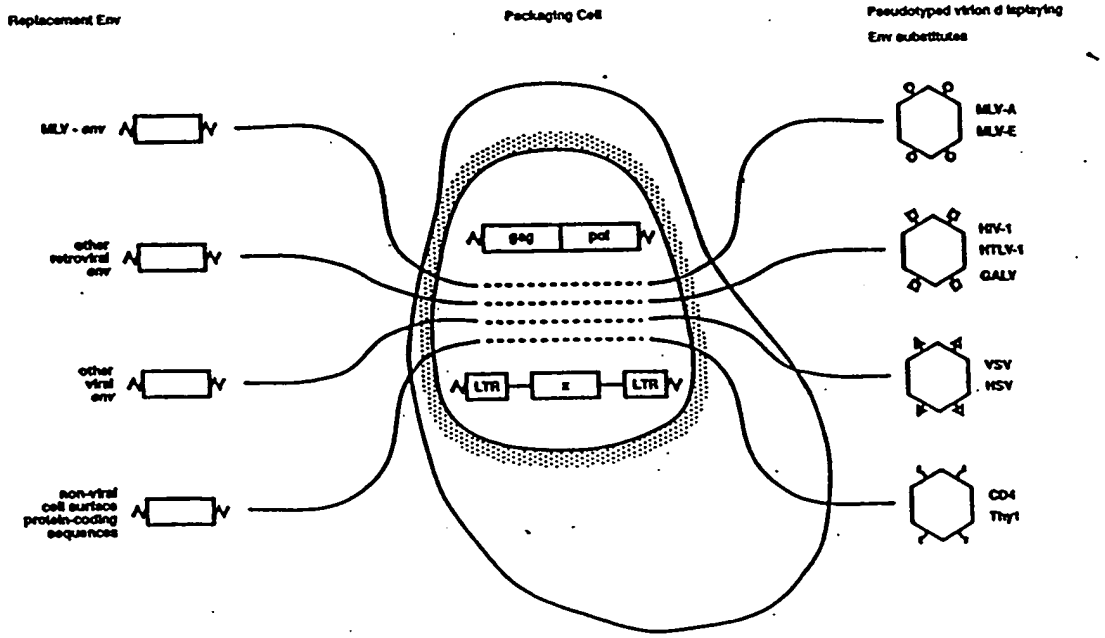

Figure 1. Generation of retroviral vectors with novel tropisms by construction of hybrid packaging lines. Transfection of a cell with genes (*gag-pol, env*) that encode viral *trans*-acting functions allows expression of all the structural components of the virion by that cell; these components can recognize and package the recombinant retroviral genome (shown here bounded by long terminal repeats (LTRs) and carrying a therapeutic gene x). Here we represent diagrammatically the various classes of retroviral pseudotypes that have been produced by providing various *env* genes in *trans;* this illustrates the principle of alteration of retroviral vector tropism by pseudotyping.

4070A to that of 10A1 (12). Engineering of murine retroviral Env proteins is being actively investigated (12–14) and is an important area of research. However, receptor recognition may involve complex interactions between the cellular ligand and different parts of the viral Env, and modification of viral tropism by direct replacement of receptor-binding sequences will not be straightforward. The function of Env proteins is not simply to adhere to host cells but also to participate in a sequence of events leading to membrane fusion. Excessive alteration of Env structure might therefore jeopardize the exp sure of hydrophobic domains required for fusion and correct viral internalization. Nevertheless, a mammalian cell tropism has been conferred on an avian retrovirus by engineering integrin-binding sequences into Env. It was found that two of the variable regions of ALV Env could be manipulated by exchanging *env* sequences with those encoding a 16-amino acid RGD-containing peptide to produce Env proteins that were processed and incorporated into retroviral particles (15). Such hybrid envelopes could still efficiently mediate infection of avian cells through the ALV receptor, and could also infect and transfer neomycin resistance to mammalian (ALV-refractory) cells that expressed RGD-recognizing integrins. Infection was not efficient and required previous deglycosylation of the virus to expose RGD epitopes, but it is an important demonstration of the principle of targeting retroviral vectors by envelope modification.

In other studies, the RSV host range has been broadened to include human cells by packaging the genome with a chimeric Env that was a fusion of the RSV signal peptide and the influenza virus hemagglutinin (16). Chimeric Env was found to be incorporated into the virions as efficiently as wild-type RSV Env. It may be possible to use influenza hemagglutinins to direct retroviral vectors to subsets of cells exhibiting particular glycosylation phenotypes as the various influenza strains possess different hemagglutinins with different precise specificities. Another candidate protein for restriction of tropism is the B19 parvovirus surface protein, the surface receptor for which has recently been characterized (17) as the tetrasaccharide of globoside (blood group P antigen), which has a very limited tissue distribution. The B19 surface protein may be susceptible to fine-tuning of saccharide specificity by recombinant techniques or site-directed mutagenesis, similar to the influenza hemagglutinin (18).

The possibility of targeting retroviral vectors to particular glycosylation phenotypes may be of special interest for cancer therapy, as many transformed cells show altered glycosylation. Whether or not any aberrantly expressed glycans can mediate viral entry is another question; a recent report indicates that retroviruses targeted to cells via lectin cross-linking cannot infect the cells after binding (19), but this could be a function of the lectin or of structural alterations caused by cross-linking rather than a function of the glycan receptor.

The demonstrable ability (16) to alter RSV tropism from avian t human cells by manipulation of envelope structure could be of great interest for cancer therapy. This is because the vast number of target cells in malignant disease suggests that either the immune system must be recruited or that a replicating vector be used to target all the tumor cells, and RSV is a replicating vector par excellence. Besides its own genome, this virus is known to carry a cell-derived oncogene; replacement f this with a therapeutic cDNA would give a replication-competent gene therapy vector.

Encouraging results have been reported using a similar approach, in which a cDNA encoding an mAb fragment capable of hapten recognition was fused to the *env* gene of MoMLV (18). Coexpression of this gene with the normal envelope in an ecotropic packaging line resulted in infective viral particles that possessed the appropriate hapten-binding activity. It should be noted that the packaging line was expressing and required parental ecotropic Env as well as the chimeric protein, so it remains to be seen if infective retroviral particles can be assembled that contain only hapten-displaying Env (20). This approach has yet to be demonstrated using a hapten directed against a relevant human antigen capable of mediating virus internalization, and is still far from in vivo application.

Targeting by retrovirus-ligand conjugates

Hepatocytes possess a unique receptor that internalizes asialoglycoproteins. Conjugation of lactose to ecotropic viral particles allowed them to be recognized as asialoglycoproteins and broadened their host range to include human hepatoma cells (21). However, this approach is limited first to cells that express the asialoglycoprotein receptor, and second to proliferating cells (because retroviruses depend on host cell mitosis in order to integrate). As normal liver cells have a very low turnover rate, this technique is most likely to be of use for in vivo delivery to malignant liver disease of the hepatocyte lineage. Furthermore, because the vector was based on an ecotropic virus, its tropism in humans would be limited entirely to hepatocytes, greatly increasing its safety compared with broad affinity vectors such as those bearing the 4070A or GALV envelope proteins.

In a more indirect approach, it was found that ecotropic MoMLV vectors bound to human hepatoma cells after being cross-linked to the transferrin receptor by a series of antibodies; however, there was no subsequent proviral integration, suggesting either that the cross-linking antibodies were inhibiting membrane fusion or that the transferrin receptor cannot mediate appropriate viral internalization (22). A similar cross-linked mAb technique has been used to target ecotropic retroviral particles to human cells in vitro by means of the streptavidin-biotin reaction (23). This allowed ecotropic virus to bind to cells expressing human class I or II MHC antigens and to become internalized and integrated. An extension of this technique (19) showed that biotinylated EGF or insulin could substitute for the anticellular receptor antibody, and that EGF and insulin receptors could mediate internalization, leading to integration, f retroviral particles bearing streptavidin-conjugated antibodies. The possibility of targeting retroviral vectors by means other than murine antibodies, which suffer from numerous disadvantages in vivo, suggests that this approach may have potential although its in vivo applicability has yet to be demonstrated.

Adenoviral vectors

Adenoviruses are double-stranded DNA viruses in which the viral genomic DNA is contained in a virally encoded protein coat (capsid) rather than a phospholipid bilayer of host cell origin. The capsid consists of three major types of subunit: the hexon, which makes up the bulk of the coat; the penton base; and the penton fiber. The fiber is attached to the capsid via the penton base and projects outward; base and fiber together are known as the penton complex. During infection, the fiber mediates initial binding of the virus t an unidentified cellular receptor and the penton base subsequently mediates virus internalization via interactions with α_v-type integrins (24). Thus, the penton complex is respon-

for binding and internalization, and therefore for viral ism at the level of cell recognition. Although adenoviral ases are usually associated in vivo with respiratory elium or the GI tract, their cellular receptors seem to be y distributed (25). Clearly then, as with retroviruses, problem is to limit viral tropism to a particular subset of es. The adenoviral proteins responsible for attachment internalization, respectively, have been well character- giving two points at which to manipulate tropism. The st promising approach is to restrict adenovirus infection e cell-binding stage by replacing the carboxyl-terminus of the fiber with a ligand conferring a particular ism, for instance, with an antibody hapten. One report) describes the restriction of adenovirus type 5 tropism by fferent kind of fiber modification where intact virions chemically modified so that their fiber carbohydrate ups were covalently linked to an asialoglycoprotein-ylysine conjugate. Such modified virus was found to have n decreased infectivity to asialoglycoprotein receptor-tive cells while retaining infectivity to receptor-positive ls. This approach would be equally applicable to targeting noviral vectors per se. It may also be possible to restrict tion by replacing the RGD-containing domain of the on base with sequences having affinity for a ligand other n RGD-recognizing integrins.

Adenoviral vectors can also be targeted via the route of administration (27); targeting of a *lacZ*-expressing adenoviral or to the kidney by renal artery or pyelic cavity infusion ulted in β-gal activity in various renal cells with no detectle expression in liver, lung, or bladder cells (27).

possible advantage of refinement of vector targeting to point of absolute specificity might be the ability to use plicating vectors for gene therapy. For cancer, development a replicating adenoviral vector, perhaps carrying a kine or suicide gene, targeted to cancer cells at the level ell binding (via fiber/base manipulations) and at the level transcription (see next section) might allow transduction h large number of malignant cells in a tumor deposit; death due to adenovirally induced lysis may even potenat the field effect of cytokines. A safety feature of such a stem would be that the immune system would be expected eventually clear such therapeutic infections (as it does for d-type infections); therefore this potential therapy only waits adequate targeting strategies.

posome vectors

ost work on targeted liposomes has been designed to leliver cyt toxic drugs to cancer cells and has been reviewed ently (28). Expression of a cDNA in the target cells makes eater demands on the vector system in that it must not lly target the appropriate cell type but also allow efficient delivery of undegraded DNA to the nucleus. For most targeted gene delivery purposes, conventional liposomes are mited because of their selective uptake by cells of the reticuloendothelial system (RES), in particular by macrophages resident in liver, spleen, and bone marrow, because of their limited extent of extravasation. Where macrophages themselves are the target, however, RES affinity is advantageous. In *L. donovani* leishmaniasis parasites not only multiply in the Kupffer cells of the liver, but are also resident in vacuol to which lysosomes fuse, so that liposomes are passively targeted not only to the parasitized cell but also to the appropriate organelle, making liposome-mediated delivery of transcriptionally targeted antisense or suicide genes to hese parasites a real possibility. It is also possible in a few ases to avoid much of the RES by the particular route of application, particularly where the target tissue is found in a discrete anatomical compartment; e.g., nontargeted liposomes could be applied directly to the bladder for treatment of carcinoma or to the lung for treatment of cystic fibrosis or αAT deficiency. Targeting by compartment has allowed confined transduction of discrete sections of arterial wall using both liposomal and retroviral vectors (29).

In most cases, however, in vivo use of liposomes requires first avoiding the RES, and second, display of appropriate tropic and fusogenic molecules (Fig. 2). Uptake by the RES can be considerably delayed, but not altogether avoided, by the use of "stealth" liposomes that display negatively charged moieties such as the ganglioside GM1 and polyethylene glycol (PEG) (28). For most systemic purposes, the stealth formula is probably essential.

Liposomes bearing an immunoglobulin complement ("immunoliposomes") can exhibit tropisms conferred by the displayed antibody. Hence, coupling to liposomes of an antibody against glioma cells increased the efficiency of gene delivery to these cells in culture by about sevenfold (30). Just as mAbs may be conjugated to liposomes to confer targeting capability, so may other ligands such as growth factors and hormones. Coupling of transferrin to liposomes followed by i.v. injection in a rabbit model resulted in significantly greater localization to bone marrow erythroblasts (31), and incorporation of surfactant protein A into liposomes increased the uptake of the liposome cargo by alveolar type II cells (32). However, it is not sufficient merely to confer upon the vector a particular binding ability; the particle must bind to a ligand that also allows fusion of liposome and cell membranes. Such considerations of appropriate internalization of vector cargo are especially important for gene delivery vectors, where the DNA must not only reach the appropriate cell type but also must reach the nucleus in undegraded form.

Conjugating virions to liposomes or incorporating viral surface glycoproteins into liposomes might create a vector that has the efficient cell attachment and entry mechanisms of a virus but not the safety drawbacks; much work has been done in this area with Sendai virus in particular (33). Another system used liposomes that displayed only the fusogenic protein of Sendai virus (F-protein) and not the cell-binding protein (hemagglutinin) (34). However, although

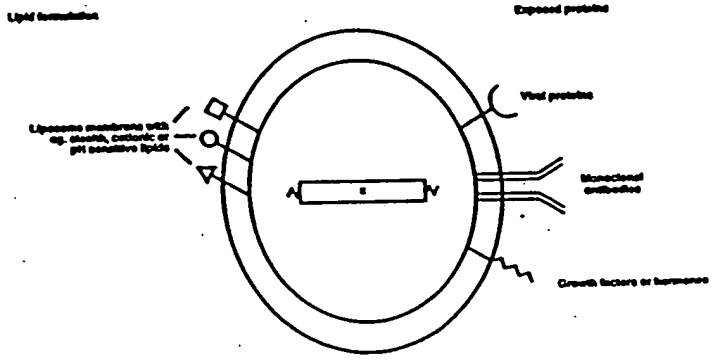

Figure 2. Modification of lipid membranes to produce targeted liposomes. Targeting of liposomes requires first abrogation of their RES affinity, and second, provision with exposed ligands having the required targeting capacity. Inclusion of ganglioside glycolipids into the lipid formulation can allow RES evasion; other lipid formulations include cationic lipids to allow promiscuous membrane binding and hence lysosome escape, and pH-sensitive lipids, which allow lysosome escape without the broad affinity conferred by cationic lipids. Various types of ligand can be inserted into the lipid membrane for provision of particular tropisms (see text for details).

such approaches can make liposomes up to 10-fold more efficient than lipofection at gene delivery (33), in terms of targeting all it can do is confer upon the liposome the tropism of the virus, and there are very few native viral receptors that exhibit a narrow and precise cell type specificity. Nevertheless, a promising system (35) is currently being developed in which respiratory epithelium is targeted by means of the surface proteins of respiratory syncytial virus (ReSV), which is responsible for infections of the lower respiratory tract. Liposome-type envelopes were constructed that displayed both the attachment and fusion proteins of ReSV, and these have been shown to enter all cells of a cultured respiratory epithelial cell line within 1 h (35).

Cationic liposomes such as the commercially produced lipofectin can efficiently avoid the lysosomal pathway because the particular lipid composition allows direct fusion of liposome and cell membranes. These particles are therefore much more efficient than conventional liposomes, and for in vitro transduction have largely replaced them. Cationic liposomes have also been used for in vivo approaches and even clinical trials; however, there seem to be no data on the extent to which these liposomes can avoid the RES, and indeed the cationic surface would seem to be incompatible with the negative charges characteristic of the stealth formulation. One report suggests that the cationic liposome has as much affinity for other cell types as for the RES after i.v. injection (36). Administration of liposomes carrying SV40-CAT resulted in widespread expression of the marker gene for up to 9 wk, albeit mainly in tissues generally associated with the RES such as spleen, liver, lymph nodes, and bone marrow as well as in vascular endothelium. CAT expression was also observed in tumor cells in this experiment, probably as a consequence of leaky tumor vasculature. It may eventually be possible to combine the efficient lysosomal avoidance of cationic liposomes with a specific targeting capacity, although the problem is likely to be that the generally fusogenic nature of cationic liposomes may preclude any precisely restricted targeting.

Molecular conjugate vectors

Targeting of plasmid DNA may be achieved by coupling the DNA to a ligand with a demonstrated cell or tissue affinity. This is usually brought about by covalently linking a polycation such as polylysine to the ligand; the polycation can then bind to and condense plasmid DNA via electrostatic interactions, leaving the ligand exposed on the surface of the conjugate (37). The ligands chosen must be efficiently endocytosed in the target cells so that DNA is efficiently internalized. One of the first receptors to be used in this way was the asialoglycoprotein receptor, whose expression is limited to hepatocytes; this receptor binds glycoproteins with terminal galactose residues for removal from the circulation; asialoorosomucoid (ASOR) is a major natural ligand for this receptor. BSA has been given specificity for the ASOR receptor by artificial galactosylation, and has been used to target CAT and human factor IX cDNAs (38) to hepatoma cells in vitro and to liver but not other tissues in vivo. Other ligands that have been used in similar conjugates include insulin (39), EGF (40), lectins (41), and transferrin (37). A major drawback of classical molecular conjugate vectors is that internalization depends on receptor-mediated endocytosis, a process that directs the receptor complex to lysosomes where it is degraded; only a small fraction of introduced

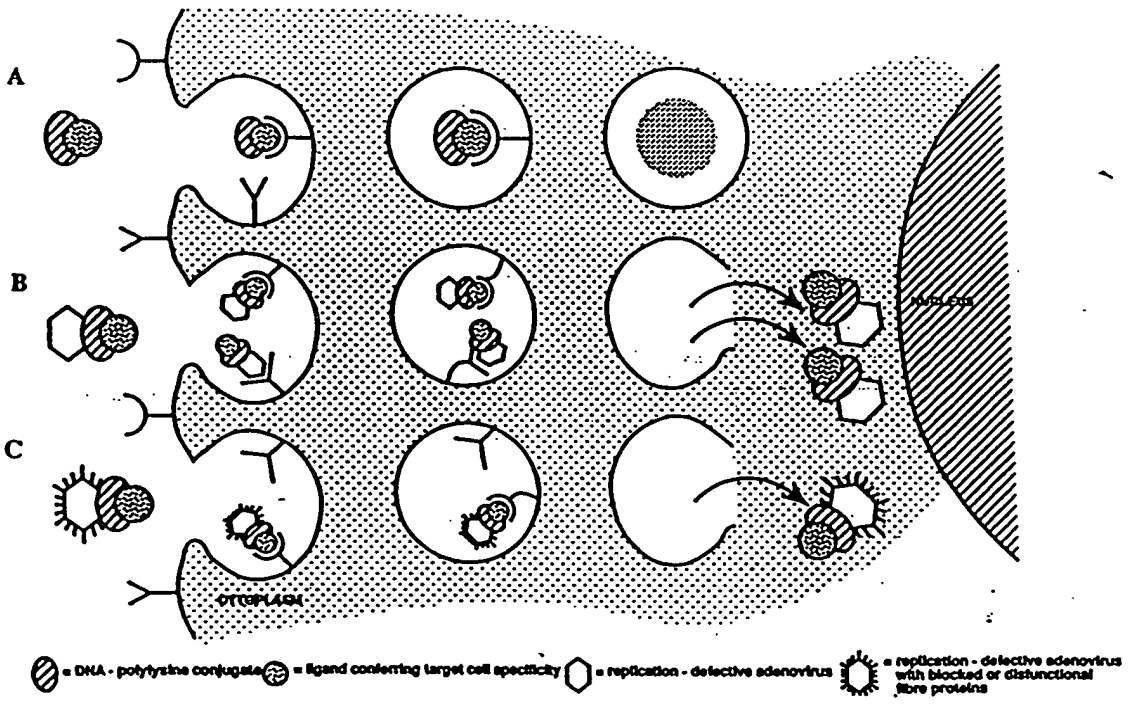

Figure 3. Targeting of plasmid DNA by molecular conjugate vectors. Conjugation of plasmid DNA to a particular ligand can confer a particular targeting capacity, but results in a vector of very low efficiency because most receptor-mediated endocytosis directs such conjugates to lysosomes where the great majority of vector DNA is degraded (route A). By complexing an adenovirus coat to the conjugate, a highly efficient vector is created by virtue of the ability of adenovirus proteins to disrupt the endosome before vector degradation (route B); however, this abrogates any targeting capacity conferred by the ligand, as the complex can enter cells either via the ligand receptor or via the virtually ubiquitous adenoviral receptor. To truly target such complexes it will be necessary to use modified adenoviral coats that retain the lysosomal escape mechanism but cannot interact with the adenoviral receptor (route C).

escapes this pathway and enters the nucleus, leading to ficiency f transduction.

w generation of molecular conjugate vectors has been ed that has the capacity to escape the degradative mal pathway by utilizing features of the adenovirus (Fig. 3). Adenovirus disrupts endosomes during cell s a consequence of a conformational change in the proteins, resulting in membrane breakdown, trig- by a drop in pH. Hence, molecular conjugate vectors red DNA to cells with greatly increased efficiency ransfection was done in the presence of adenovirus. er, this effect relies on both virus and vector being it in the same endosome. To improve efficiency, the virus has been coupled directly to the molecular con- (37). However, adenovirus receptors are virtually ous and so the coupling of an adenovirus receptor to eted molecular conjugate would be expected to par- r completely abrogate any preferential tropism con- by the ligand. Blocking the interaction of fiber with virus receptor by mAb to the fiber resulted (42) in a r that was both targeted to a specific subset of cells and escape the lysosomal pathway. A more satisfactory ap- would be to create recombinant adenoviral vectors isplay dysfunctional fiber proteins in order to bypass ntibody-coating step.

in vivo experiments have been attempted using virus-molecular conjugate complexes, and in fact it is ely that such vectors will be routinely applicable to in work, although they are likely to be of use for ex vivo gies (43). This is a consequence first of the size of the ex (transferrin-polycation conjugates are approxi- ly 100 nm in diameter (44); complexed with AdV they d be even larger), which will prohibit extensive extrava- or tissue penetration, and second, of the likelihood of immunogenicity of the AdV proteins (45).

GETING OF GENE THERAPY VECTORS AT THE ETIC LEVEL

nscripti nal targeting

apeutic cDNAs may be limited in expression to a partic- subset of cells by placing them under the control of ılat ry elements that possess binding sites for tissue- icted positive or negative *trans*-acting factors (Fig. 4). ectly regulated expression may require, in addition to 5' moter sequences, distant elements either 5' or 3' to the ing regi n; these elements act together with the promoter allow tissue-specific expression at appropriate levels in- ndent f position of integration. Such locus control ions (LCRs) have been identified for a number of genes. Rs would be of much use for gene augmentation but the sfer of such large sections of DNA to target cells will be blematic, particularly in vivo, and in fact for the foreseea- future may be confined to ex vivo strategies. Where a nogenic defect results in pathology in more than one tis- the most pragmatic approach to appropriately limit the ressi n of therapeutic cDNA is to use the cellular omoter/enhancer elements native to the defective gene. rthermore, the use of cellular rather than viral promoters ces the chance of loss of cDNA expression due to inacti- on f viral sequences by methylation or other mechan- ns (46). Thus, cellular promoters may confer benefits both long-term expression and of tissue-restricted expression, where vector-targeting at the cell-binding level has not n achieved it may represent the only way of limiting ex- ession of exogenous cDNA.

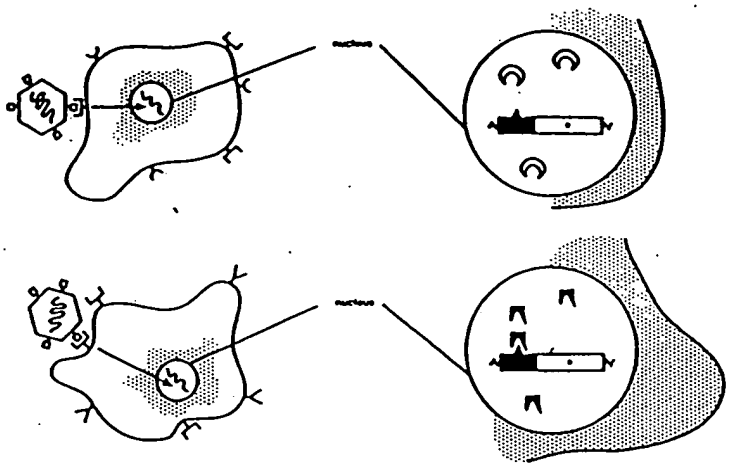

Figure 4. Tissue-restricted transcription. A promiscuously binding vector can be targeted at the transcriptional level if the therapeutic gene (x) is controlled by 5' regulatory elements (shown here as a shaded region upstream of x) active only in the presence of tissue-specific nuclear transcription factors; thus expression of x occurs only in the target cells.

Tissue-specific cellular regulatory elements have great potential for development of safe, targeted vectors for gene therapy. For example, the creatine kinase promoter has been used in a plasmid vector to restrict dystrophin cDNA expression to skeletal and cardiac muscle, and in the *mdx* mouse model of Duchenne muscular dystrophy, mice transgenic for this promoter-cDNA construct were found to exhibit correction of dystrophic symptoms (47). A potential approach to the treatment of B cell lymphoma involves expression of suicide genes transcriptionally regulated by promoter/enhancers from the Ig heavy chain or the *x* light chain genes; expression plasmids containing the diphtheria toxin A (DT-A) gene controlled by these regulatory elements mediated significant expression of DT-A in B lymphoid cells but not in HeLa cells or fibroblasts (48).

Endothelial cells are attractive recipients for gene transfer therapies not only for obvious purposes such as targeting of tumor vasculature or therapy of cardiovascular disease, but also for the systemic secretion of therapeutic factors. An endothelial cell-specific regulatory region has recently been characterized (49) as 500 bp of 5' sequences, associated with the gene for von Willebrand's factor, acting in conjunction with an essential region in the first intron. This promoter could be particularly useful when driving a suicide gene in a retroviral vector as it would then be targeted to dividing endothelial cells, i.e., almost exclusively tumor vasculature.

Tissue-specific cellular promoters frequently retain their specificity in the context of a retroviral vector (50); however, this is not always the case, and the design of the retroviral vector may have significant effects on tissue specificity due to promoter interference (51). Tissue-specific promoters have also been shown to appropriately restrict cDNA expression in the context of recombinant adenoviruses, e.g., the rat albumin promoter maintained its hepatoma cell specificity in vitro (52), albeit at low levels.

Antiviral therapy using transcriptional targeting

Transcripti nal targeting may be of particular use in the therapy of particular kinds of viral infection. In cases where the viral life cycle depends on self-encoded autoregulatory

proteins, vectors can be made in which therapeutic cDNAs are transcriptionally regulated by these same viral proteins. Transcription of the therapeutic cDNA is therefore limited to cells that are infected by the virus, and thus such an approach could be either prophylactic or curative. This strategy has been applied to experimental HIV therapies. One recent report (53) described the constructi n of a recombinant retrovirus containing HSV-TK driven by the HIV-2 LTR-TAR; cells expressing this construct became susceptible to ganciclovir after infection by HIV-2 in vitro.

Targeting proliferating cells

Murine C-type retroviral vectors can combine the ability to express cDNA from an internal tissue-specific promoter with an innate tropism for proliferating tissue. Therefore, they have great potential as vectors for the gene therapy of cancer, because restricted cDNA expression is of particular importanc in strategies that involve delivery of cytokine or suicide genes and malignancies are often distinguished by rapid division in a relatively quiescent background. Indeed, in a very few cases the retroviral requirement for cell division may be sufficient in itself to target the therapy (Fig. 5); where tumors arise in the CNS their high rate of proliferation in the context of a completely postmitotic tissue, in an anatomical compartment that is separated from the rest of the body, allows fficient targeting with retroviral vectors (54). As an additional targeting feature for malignancies of the CNS, the glial-specific promoter region of the mouse myelin basic protein gene has been used to drive HSV-TK in a retroviral vector (55); this approach could allow long-term administration of producer cells at the primary site or systemic vector application to treat metastatic deposits as collateral infection f nonglial cells would not result in expression f th suicide gene.

Retroviral vectors would also be useful in targeting liver malignancies, as the liver is also slowly proliferative under normal circumstances. Tissue-specific promoters would be essential for such strategies, because unlike the CNS, the liver is not efficiently insulated from the rest of the body. Amphotropic retroviral vectors have been constructed carrying HSV-TK cDNA driven either by the albumin or the a-fetoprotein promoters (56). The albumin promoter was active only in cells of the liver lineage; the a-fetoprotein promoter conferred an extra level of targeting in that it was hepatoma-specific as opposed to hepatocyte-specific (a-fetoprotein is normally expressed only in fetal tissues).

The 5' region of the tyrosinase gene has also been used to restrict expression of therapeutic cDNAs to melanocytes and melanoma cells both in vitro and in vivo by means of retroviral vectors (51, 57). This kind of transcriptional targeting may be useful in VDEPT approaches for melanoma because normal melanocytes are dispersed and of low density in body tissues, and their ablation is likely to be minimally pathological. Even better would be the usurpation of tumor-specific transcriptional regulation by using promoter sequences from genes whose overexpression is limited to transformed tissue. One such candidate is the oncogene ERBB2, which is overexpressed in a variety of tumors. The ERBB2 promoter sequences have been used to drive cytosin deaminase cDNA in a retroviral vector (58); this strategy conferred sensitivity to ERBB2-overproducing cells but not to control cells, and represents a potentially widely applicable method of tumor-preferential transcriptional targeting. The a-fetoprotein promoter is in effect completely tumor-specific, but is applicable only to malignancies of the liver.

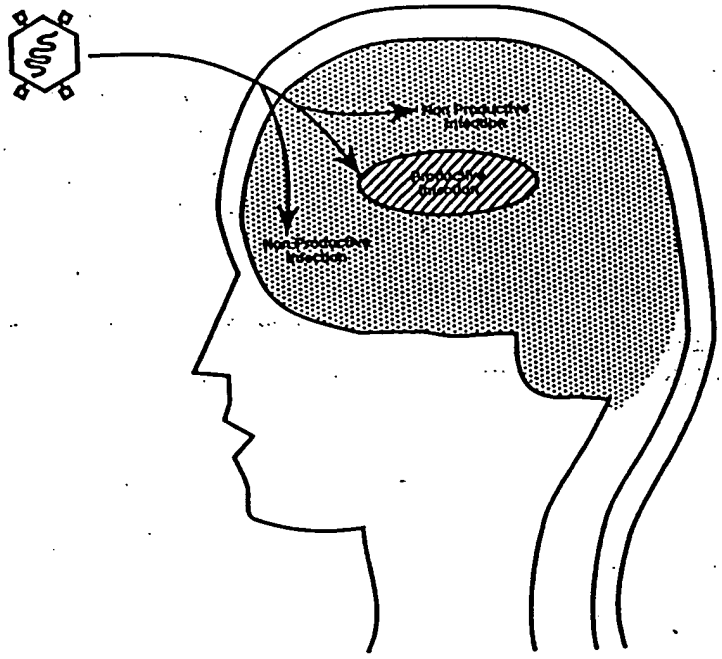

Figure 5. Targeting proliferating cells. Retroviral vectors require cell division for integration and gene expression; therefore where a tumor arises in a completely postmitotic background, such as the CNS, the proliferation of the malignant tissue may be sufficient in itself to allow efficiently targeted delivery of suicide genes via recombinant retroviruses. Actively replicating (tumor) cells are represented by diagonal lines; quiescent neuron tissue is represented by dots.

Exploitation of natural viral tropisms

An obvious approach to the precise targeting of tissues is to make vectors from viruses that have preferential patterns of transcription in target tissues, such as HSV vectors for nervous tissue. However, careful dissection of the genomes of these viruses will be necessary to separate pathogenic sequences from those that confer transcriptional specificity; in most cases it will be preferable to use cellular promoters in the vector of choice, especially as the range of transcripti nally targeted viral genomes is not great.

There may be one remarkable exception to the general requirement for cellular promoters rather than viral prom ters in gene therapy, namely, the use of autonomous parvoviral sequences for targeting transformed cells (see ref 59 for review). These viruses preferentially kill transformed cells (60), and coinjection of mouse minute virus (MVM) and Ehrlich ascites tumor cells into the peritoneal cavities of mice inhibited tumor formation by up to 90%. Furthermore, mice that had survived one such coinjection were resistant to a second tumor challenge 5–6 wk later. The precise basis of parvovirus oncotropism is not understood but may be related t an effect of the transformed cell environment on the production or activity of parvovirus autoregulatory proteins. The parvovirus promoter that is preferentially transactivated in certain transformed cells is clearly a candidate to control transcription of suicide or cytokine genes in parvovirus vectors for cancer th rapies. Recombinant parvovirus vectors have be n mad and shown to both transfer exogenous cDNA expression to recipient cells and retain their oncotropism in vitro (61) for human and murine cells. Recombinant parvoviruses may therefore represent one f the most promising approaches t cancer therapies for the future.

Targeted integration: site-specific recombination

Nonintegrating vectors are adequate for transient expression of cDNA. Where the object is a "one-shot" treatment for cure of a genetic disorder, it is necessary to use either an integrating vector or a stably replicating extrachromosomal element. For the future, sequences containing mammalian origins of replication or even entire mammalian artificial chromosomes (62) could have great potential especially for ex vivo approaches. Similarly, vectors based on the Epstein-Barr virus, which is stably maintained episomally as a plasmid in human cells, may one day be suitable for clinical use.

The ideal approach would be to target the exogenous DNA to the mutant gene, i.e., gene replacement rather than gene augmentation. Such gene targeting approaches may be of use for ex vivo strategies to stably transduce cells with less likelihood of simultaneous transformation (63). Such in vitro homologous recombination may be useful in inactivating genes responsible for MHC class I expression in myoblasts to create a universal carrier cell that can be transplanted regardless of the recipient HLA type (63). This approach is applicable to any ex vivo strategy that requires implantation of viable transduced but otherwise unchanged cells. The technology required to accomplish this at levels of efficiency relevant to in vivo gene transfer does not yet exist and so integrating gene therapy vectors at present can offer only gene augmentation.

Nontargeted integration could be hazardous if completely random, not only by turning on downstream oncogenes via promoter readthrough but also by direct disruption of genes, and this is the main source of concern with regard to the use of retroviral vectors in humans. Vectors with the capacity for site-specific integration would overcome these problems. Adeno-associated virus is a defective parvovirus that potentially is widely applicable in gene transfer strategies because it is tropic for many cell types, nonpathogenic in humans (in the absence of helper virus the AAV genome does not replicate but integrates into the genome and assumes a state of latency), and can be manipulated to derive recombinant genomes capable of vectoring exogenous DNA (64). Although these vectors can package only up to 4.5 kb as compared with the retrovirus limit of approximately 7 kb, they are said to have one major advantage over other integrating vectors, namely, a propensity (which is far from total) for apparently harmless integration into a region of human chromosome 19 known as AAVS1 (see review, ref 65). Where such specific integration occurs, it is almost certainly mediated by virally encoded proteins with affinity both for the target site and for the virus genome (66). Although integrated viral sequences remain dormant until superinfection by AdV/HSV, exogenous cDNAs driven by internal promoters can still be active (furthermore, the transcriptional inactivity of the viral ITR means that there will be no promoter interference leading to, for example, loss of tissue specificity of exogenous promotor, and less chance of insertional mutagenesis for the same reason). Thus AAV vectors have been shown to confer neomycin resistance and in some cases to integrate with site specificity (64). This study also showed that AAV vectors preserved their site specificity after transfection in plasmid form; the use of a transfectable plasmid rather than a viral vector might overcome the packaging limitations of AAV vectors (64). It must be said, however, that some groups report that recombinant AAV vectors show site specificity in only a relatively minor proportion of the total number of integration events. There have been several attempts to explore the therapeutic potential of AAV vectors, e.g., the delivery of cDNA for the correction of the cystic fibrosis defects (67).

There may be other vector systems also capable of site-specific integration. Eukaryotic genomes harbor large numbers of endogenous transposable elements of various types (68), i.e., autonomously replicating units that can insert themselves into the host genome. Some of these elements, known as LTR retrotransposons, are very similar to retroviruses both in replication cycle and in organization, being bound by LTRs and possessing coding regions with homology to retroviral *gag-pol* genes. The replicative cycle of LTR retrotransposons exactly parallels that of the retroviruses except that there is no envelope stage, thus, cytoplasmic virus-like particles (69) are formed containing reverse transcriptase, the RNA form of the retrotransposon, and cellular tRNA primers for reverse transcription. Such elements include *copia*, yeast Ty, and the intracisternal A particle of mice; clearly they have great potential as vectors of improved safety as their use with retroviral packaging lines would be less likely to result in helper virus production through homologous recombination. Indeed a mouse retrotransposon VL30 has already been made into a gene transfer vector (70), which can be produced in a standard retroviral packaging line. Endogenous retrotransposons a priori would be expected, through coevolution with the host genome, to display a degree of site specificity of integration as continuous random retrotransposition would be deleterious to the cell. Yeast retrotransposons offer the best examples of site-specific retrotransposons, and moreover, their site of integration appears to be benign. Two of the five *Saccharomyces cerevisiae* retrotransposons, Ty1 and Ty3, exhibit unambiguous site specificity of integration (71). Ty3 elements integrate into sites upstream of genes transcribed by RNA pol III, frequently within 1–4 nucleotides of the start site of transcription. It has been suggested that this sequence-independent site specificity is brought about by interaction of the retrotransposon with elements involved in RNA pol III-mediated transcription, e.g., TFIIIB (71). Similarly, Ty1 preferentially integrates upstream of tRNA genes (71) 57% of insertions occurring within 400 bp of a tRNA gene. A consequence of this specificity is that yeast genes are only rarely interrupted by Ty1 insertions as regions upstream of yeast tRNA genes rarely contain open reading frames (71). The great similarity of LTR retrotransposons to retroviruses allows them to be made into vectors with conventional retrovirus packaging lines (70); possibly the development of a packaging line that provides retrotransposon rather than retroviral *gag-pol* in *trans* will allow the production of vectors with integrational site specificity.

SUMMARY AND PERSPECTIVES

Of the gene therapy protocols that have so far entered clinical trials, targeting of the appropriate vectors has been achieved largely only by indirect means. Thus, several such trials (for example, for treatment of ADA deficiency, HIV infection, or cancer) have used specific cell populations that have been removed from the patient and infected in vitro by nontargeted amphotropic retroviruses before being returned in vivo. Further levels of targeting have been achieved in some cases by careful choice of the patient's cells; for instance, ex vivo transduction of tumor infiltrating lymphocytes with potentially tumoricidal genes has been proposed as a means of delivering their products to tumor deposits at much higher concentrations than would otherwise be possible.

In contrast to ex vivo manipulation of target cells where the vector requires very little, if any, intrinsic targeting capability, there are an increasing number of protocols in which

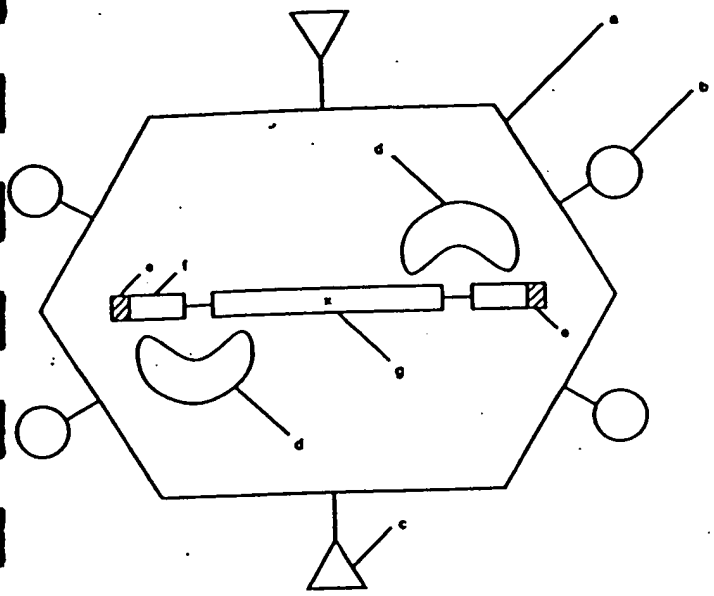

Figure 6. A theoretical composite vector. Some features that might be incorporated in an ideal synthetic vector include a stable, nonimmunogenic envelope, probably lipid (*a*); exposed ligands to confer a particular affinity on the vector (*b*); moieties that encourage fusion between vector and target cell membranes (*c*); proteins to allow directed integration of vector DNA, e.g., site-specific recombinases (*d*); sequences to enable homologous recombination between vector DNA and particular loci of the target genome (*e*); tissue-specific promoter regions to allow restricted expression of the therapeutic gene (*f*); and the therapeutic cDNA (*g*).

recombinant genes are delivered directly to patients in vivo (such as for the treatment of cystic fibrosis and cancer). Once again, targeting at the level of the vector has not yet been particularly well developed; hence, liposome or viral-mediated delivery of the CFTR gene to airway epithelial cells of CF patients has relied largely on the localized delivery of the vectors directly to the affected tissues, and on the fact that there is good evidence that inadvertant expression of the CFTR gene in cells other than the target epithelial cells may have few adverse effects. Localized delivery has also been used in the treatment of brain tumor deposits, using stereotactic injection of retroviral producer cells, but with the added sophistication that the retroviruses would be expected to infect only the actively dividing tumor cells and n t the surrounding neural tissue.

However, for the long-term success as well as the widespread applicability of human gene therapy, there will have to be advances in the ability with which clinicians can confidently administer recombinant vectors for the treatment of genetic disease directly to affected tissues in vivo. For this to occur, many targeting strategies outlined in this review, which are currently only at the experimental level, will have to be translated into components of safe and highly efficient delivery systems. Vectors have already been developed that incorporate transcriptional specificity for a certain tissue type; however, the development of surface targeting has been more problematic in most cases. The biggest challenge for the next 5 years will be to combine targeting with efficiency in the production of the vector systems of the future. So far, attainment of one usually compromises the th r; for example, we have constructed retroviral vectors targeted at the level of transcription to melanoma cells but these viruses are generally of lower titer than their nontarget d counterparts.

Nonetheless, the imagination and the technology is currently available to allow us to hope that vectors will eventually be constructed that can include both efficiency and specificity. In particular, it does not seem unrealistic to suppose that the gene therapy vectors of th future will not be based exclusively on any single virus or physical vector system alone but will be synthetic, custom-designed vehicles (Fig. 6) into which specific targeting features can be included depending on the particular clinical requirements of the target disease and tissue. FJ

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REFERENCES

1. Weiss, R. A. (1993) Cellular receptors and viral glycoproteins involved in retrovirus entry. In *The Retroviridae* (Levy, J. A., ed) pp. 1-108, Plenum, New York
2. Dalgleish, A. G., Beverley, P. C. L., Clapham, P. R., Crawford, D. H., Greaves, M. F., and Weiss, R. A. (1984) The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. *Nature* 312, 763
3. Poznansky, M., Lever, A., Bergeron, L., Haseltine, W., and Sodroski, J. (1991) Gene transfer into human lymphocytes by a defective human immunodeficiency virus type 1 vector. *J. Virol.* 65, 532-536
4. Toggas, S. M., Masliah, E., Rockenstein, E. M., Rall, G. F., Abraham, C. R., and Mucke, L. (1994) Central nervous system damage produced by expression of the HIV-1 coat protein gp120 in transgenic mice. *Nature* 367, 188-193
5. Miller, A. D. (1992) Retroviral vectors. *Curr. Top. Microbiol. Immunol.* 158, 1-24
6. Landau, N. R., Page, K. A., and Littman, D. R. (1991) Pseudotyping with human T-cell leukemia virus type I broadens the human immunodeficiency virus host range. *J. Virol.* 65, 162-169
7. Takeuchi, Y., Simpson, G., Vile, R. G., Weiss, R. A., and Collins, M. K. L. (1992) Retroviral pseudotypes produced by rescue of a Moloney murine leukemia virus vector by C-type, but not D-type, retroviruses. *Virology* 186, 792-794
8. Vile, R. G., Schulz, T. F., Danos, O. F., Collins, M. K. L., and Weiss, R. A. (1991) A murine cell line producing HTLV-I pseudotype virions carrying a selectable marker gene. *Virology* 180, 420-424
9. Zhu, Z., Chen, S. S. L., and Huang, A. S. (1990) Phenotypic mixing between human immunodeficiency virus and vesicular stomatitis virus or herpes simplex virus. *J. AIDS* 3, 215-219
10. Calafat, J., Janssen, H., Demant, P., Hilgers, J., and Zavada, J. (1983) Specific selection of host cell glycoproteins during assembly of murine leukaemia virus and vesicular stomatitis virus. *J. Gen. Virol.* 64, 1241-1253
11. Young, J. A. T., Bates, P., Willert, K., and Varmus, H. E. (1990) Efficient incorporation of human CD4 protein into avian leukosis virus particles. *Science* 250, 1421-1423
12. Ott, D., and Rein, A. (1992) Basis for receptor specificity of nonecotropic murine leukemia virus surface glycoprotein $gp70^{SU}$. *J. Virol.* 66, 4632-4638
13. Battini, J.-L., Heard, J. M., and Danos, O. (1992) Receptor choice determinants in the envelope glycoproteins of amphotropic, xenotropic and polytropic murine leukemia viruses. *J. Virol.* 66, 1468-1475
14. Morgan, R. A., Nussbaum, O., Muenchau, D. D., Shu, L., Couture, L., and French Anderson, W. (1993) Analysis of the functional and host range-determining regions of the murine ecotropic and amphotropic retrovirus envelope proteins. *J. Virol.* 67, 4712-4721
15. Valsesia-Wittman, S., Drynda, A., Deleage, G., Aumailley, M., Heard, J.-M., Danos, O., Verdier, G., and Cosset, F.-L. (1994) Modifications in the binding domain of avian retrovirus envelope protein to redirect the host range of retroviral vectors. *J. Virol.* 68, 4609-4619
16. Dong, J., Roth, M. G., and Hunter, E. (1992) A chimeric avian retrovirus containing the influenza virus hemagglutinin gene has an expanded host range. *J. Virol.* 66, 7374-7382
17. Brown, K. E., Anderson, S. M., and Young, N. S. (1993) Erythrocyte P antigen: cellular receptor for B19 parvovirus. *Science* 262, 114-117
18. Rogers, G. N., Paulson, J. C., Daniels, R. S., Skehel, J. J., Wilson, I. A., and Wiley, D. C. (1983) Single amino acid substitutions in influenza haemagglutinin change receptor-binding specificity. *Nature* 304, 76-78
19. Etienne-Julan, M., Roux, P., Carillo, S., Jeanteur, P., and Piechaczyk, M. (2222) The efficiency of cell targeting by recombinant retroviruses depends on the nature of the receptor and the composition of the artificial cell-virus linker. *J. Gen. Virol.* 73, 3251-3255
20. Russell, S. J., Hawkins, R. E., and Winter, G. (1993) Retroviral vectors displaying functional antibody fragments. *NAR* 21, 1081-1085
21. Neda, H., Wu, C. H., and Wu, G. Y. (1991) Chemical modification of an ecotropic murine leukemia virus results in redirection of its target cell specificity. *J. Biol. Chem.* 266, 14143-14146

Goud, B., Legrain, P., and Buttin, G. (1988) Antibody-mediated binding of a murine ecotropic Moloney retroviral vector to human cells allows internalisation but not the establishment of the proviral state. *Virology* 163, 251-254

Roux, P., Jeanteur, P., and Piechaczyk, M. (1989) A versatile and potentially general approach to the targeting of specific cell types by retroviruses: application to the infection of human cells by means of major histocompatibility complex class I and class II antigens by mouse ecotropic murine leukemia virus-derived viruses. *Proc. Natl. Acad. Sci. USA* 86, 9079-9083

Wickham, T. J., Mathias, P., Cheresh, D. A., and Nemerow, G. R. (1993) Integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ promote adenovirus internalisation but not virus attachment. *Cell* 73, 309-319

Stratford-Perricaudet, L. D., Makeh, I., Perricaudet, M., and Briand, P. (1992) Widespread long-term gene transfer to mouse skeletal muscles and heart. *J. Clin. Invest.* 90, 626-6330

Wu, G. Y., Zhan, P., Sze, L. L., Rosenberg, A. R., and Wu, C. H. (1994) Incorporation of adenovirus into a ligand-based DNA carrier system results in retention of original receptor specificity and enhances targeted gene expression. *J. Biol. Chem.* 269, 11542-11546

Moullier, P., Friedlander, G., Calise, D., Ronco, P., Perricaudet, M., and Ferry, N. (1994) Adenoviral-mediated gene transfer to renal tubular cells in vivo. *Kidney Int.* 45, 1220-1225

Allen, T. M. (1994) Long-circulating (sterically stabilised) liposomes for targeted drug delivery. *TIPS* 15, 215-220

Nabel, E. G., Plautz, G., and Nabel, G. J. (1990) Site-specific gene expression in vivo by direct gene transfer into the arterial wall. *Science* 249, 1285-1288

Mizuno, M., Yoshida, J., Sugita, K., Inoue, I., Seo, H., Hayashi, Y., Koshizaka, T., and Yagi, K. (1990) Growth inhibition of glioma cells transfected with the human β-interferon gene by liposomes coupled with a monoclonal antibody. *Cancer Res.* 50, 7826-7829

Stavridis, J. C., Deliconstantinos, G., Psallidopoulos, M. C., Armenakas, N. A., Hadjiminas, D. J., and Hadliminas, J. (1986) Construction of transferrin-coated liposomes for in vivo transport of exogenous DNA to bone marrow erythroblasts in rabbits. *Exp. Cell Res.* 164, 568-572

Walther, F. J., David-Cu, R., Supnet, M. C., Longo, M. L., Fan, B. R., and Bruni, R. (1993) Uptake of antioxidants in surfactant liposomes is enhanced by SP-A. *Am. J. Physiol.* 265, L330-L339

Morishita, R., Gibbons, G. H., Kaneda, Y., Ogihara, T., and Dzau, V. J. (1993) Novel in vitro gene transfer method for study of local modulators in vascular smooth muscle cells. *Hypertension* 21, 894-899

Bagai, S., and Sarkar, D. P. (1993) Targeted delivery of hygromycin B using reconstituted Sendai viral envelopes lacking haemagglutinin-neuraminidase. *FEBS Lett.* 326, 183-188

Schreier, H., Gonzalez-Rothi, R. J., and Steeenko, A. A. (1993) Pulmonary delivery of liposomes. *J. Contr. Release* 24, 209-223

Zhu, N., Liggitt, D., Liu, Y., and Debs, R. (1993) Systemic gene expression after intravenous DNA delivery into adult mice. *Science* 261, 209-211

Michael, S. I., and Curiel, D. T. (1994) Strategies to achieve targeted gene delivery via the receptor-mediated endocytosis pathway. *Gene Ther.* 1, 223-232

Ferkol, T., Lindberg, G. L., Chen, J., Perales, J. C., Crawford, D. R., Ratnoff, O. D., and Hanson, R. W. (1993) Regulation of the phosphoenolpyruvate carboxykinase/human factor IX gene introduced into the livers of adult rats by receptor-mediated gene transfer. *FASEB J.* 7, 1081-1091

Rosenkranz, A. A., Yachmenev, S. V., Jans, D. A., Serebryakova, N. V., Murav'ev, V. I., Peters, R., and Sobolev, A. S. (1992) Receptor-mediated endocytosis and nuclear transport of a transfecting DNA construct. *Exp. Cell Res.* 199, 323-329

Chen, J., Gamou, S., Takayanagi, A., and Shimizu, N. (1994) A novel gene delivery system using EGF receptor-mediated endocytosis. *FEBS Lett.* 338, 167-169

Batra, R. K., Wang-Johanning, F., Wagner, E., Garver, R. I., and Curiel, D. T. (1994) Receptor-mediated gene delivery employing lectin-binding specificity. *Gene Ther.* 1, 255-260

Michael, S. I., Huang, C.-H., Romer, M. U., Wagner, E., Hu, P.-C., and Curiel, D. T. (1993) Binding-incompetent adenovirus facilitates molecular conjugate-mediated gene transfer by the receptor-mediated endocytosis pathway. *J. Biol. Chem.* 268, 6866-6869

Lozier, J. N., Thompson, A. R., Hu, P.-C., Read, M., and Brinkhouse, K. M. (1994) Efficient transfection of primary cells in a canine hemophilia B model using adenovirus-polylysine-DNA conjugates. *Hum. Gene Ther.* 5, 313-322

Wagner, E., Zenke, M., Cotten, M., Beug, H., and Birnstiel, M. L. (1990) Transferrin-polycation conjugates as carriers for DNA uptake into cells. *Proc. Natl. Acad. Sci. USA* 87, 3410-3414

Yang, Y., Nunes, F. A., Berencsi, K., Furth, E., Gonczol, E., and Wilson, J. M. (1994) Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy. *Proc. Natl. Acad. Sci. USA* 91, 4407-4411

Hoeben, R. C., Migchielson, A. A. J., van der Jagt, R. C. M., van Ormondt, H., and van der Eb, A. J. (1991) Inactivation of the Moloney murine leukemia virus long terminal repeat in murine fibroblast cell lines is associated with methylation and is dependent on its chromosomal position. *J. Virol.* 65, 904-912

Cox, G. A., Cole, N. M., Matsumura, K., Phelps, S. F., Hauschka, S. D., Campbell, K. P., Faulkner, J. A., and Chamberlain, J. S. (1993) Overexpression of dystrophin in transgenic mdx mice eliminates dystrophic symptoms without toxicity. *Nature* 364, 725-729

Maxwell, I. H., Glode, L. M., and Maxwell, F. (1991) Expression of the diphtheria toxin A-chain coding sequence under the control of promoters and enhancers from immunoglobulin genes as a means of directing toxicity to B-lymphoid cells. *Cancer Res.* 51, 4299-4304

Jahroudi, N., and Lynch, D. C. (1994) Endothelial-cell-specific regulation of von Willebrand factor gene expression. *Mol. Cell. Biol.* 14, 999-1008

Hatzoglou, M., Lamers, W., Bosch, F., Wynshaw-Boris, A., Clapp, D. W., and Hanson, R. W. (1990) Hepatic gene transfer in animals using retroviruses containing the promoter from the gene for phosphoenolpyruvate carboxykinase. *J. Biol. Chem.* 265, 17285-17293

Vile, R. G., Miller, N., Chernajovsky, U., and Hart, I. R. (1994) A comparison of the properties of different retroviral vectors containing the murine tyrosinase promoter to achieve transcriptionally targeted expression of the HSVtk or IL-2 genes. *Gene Ther.* 1, 1-10

Friedman, J. M., Babiss, L. E., Clayton, D. F., and Darnell, J. E. (1986) Cellular promoters incorporated into the adenovirus genome: cell specificity of albumin and immunoglobulin expression. *Mol. Cell. Biol.* 6, 3791-3797

Brady, H. J. M., Miles, C. G., Pennington, D. J., and Dzierzak, E. A. (1994) Specific ablation of human immunodeficiency virus Tat-expressing cells. *Proc. Natl. Acad. Sci. USA* 91, 365-369

Culver, K. W., Ram, Z., Wallbridge, S., Ishii, H., Oldfield, E. H., and Blaese, R. M. (1992) In vivo gene transfer with retroviral vector-producer cells for treatment of experimental brain tumours. *Science* 256, 1550-1552

Miyao, Y., Shimizu, K., Moriuchi, S., Yamada, M., Nakahira, K., Nakajima, K., Nakao, J., Kuriyama, S., Tsujii, T., Mikoshiba, K., Hayakawa, T., and Ikenaka, K. (1993) Selective expression of foreign genes in glioma cells: use of the mouse myelin basic gene promoter to direct toxic gene expression. *J. Neurosci. Res.* 36, 472-479

Huber, B. E., Richards, C. A., and Krenitsky, T. A. (1991) Retroviral-mediated gene therapy for the treatment of hepatocellular carcinoma: an innovative approach for cancer therapy. *Proc. Natl. Acad. Sci. USA* 88, 8039-8043

Vile, R. G., and Hart, I. R. (1993) Use of tissue-specific expression of the herpes simplex virus thymidine kinase gene to inhibit growth of established murine melanomas following direct intra-tumoral injection of DNA. *Cancer Res.* 53, 3860-3864

Harris, J. D., Gutierrez, A. A., Hurst, H. C., Sikora, K., and Lemoine, N. R. (1994) Gene therapy for cancer using tumour-specific prodrug activation. *Gene Ther.* 1, 170-175

Mayor, H. D. (1993) Defective parvoviruses may be good for your health. *Prog. Med. Virol.* 40, 193-205

Guetta, E., Graziani, Y., and Tal, J. (1986) Suppression of Ehrlich ascites tumors in mice by minute virus of mice. *J. Natl. Cancer Inst.* 76, 1177-1180

Dupont, F., Tenenbaum, L., Guo, L.-P., Spegelaere, P., Zeicher, M., and Rommelaere, J. (1994) Use of an autonomous parvovirus vector for selective transfer of a foreign gene into transformed human cells of different origin and its expression therein. *J. Virol.* 68, 1397-1406

Huxley, C. (1994) Mammalian artificial chromosomes: a new tool for gene therapy. *Gene Ther.* 1, 7-12

Arbones, M. L., Austin, H. A., Capon, D. J., and Greenburg, G. (1994) Gene targeting in normal somatic cells: inactivation of the interferon-γ receptor in myoblasts. *Nature Genet.* 6, 90-97

Shelling, A. N., and Smith, M. G. (1994) Targeted integration of transfected and infected adeno-associated virus vectors containing the neomycin resistance gene. *Gene Ther.* 1, 165-169

Samulski, R. J. (1993) Adeno-associated virus: integration at a specific chromosomal locus. *Curr. Opin. Genet. Dev.* 3, 74-80

Chiorini, J. A., Weitzman, M. D., Owens, R. A., Urcelay, E., Safer, B., and Kotin, R. M. (1994) Biologically active Rep proteins of adeno-associated virus type 2 produced as fusion proteins in *Escherichia coli*. *J. Virol.* 68, 797-804

Flotte, T. R., Afione, S. A., Conrad, C., McGrath, S. A., Solow, R., Oka, H., Zeitlin, P. L., Guggino, W. B., and Carter, B. J. (1993) Stable in vivo expression of the cystic fibrosis transmembrane conductance regulator with an adeno-associated virus vector. *Proc. Natl. Acad. Sci. USA* 90, 10613-10617

McDonald, J. F. (1993) Evolution and consequences of transposable elements. *Curr. Opin. Genet. Dev.* 3, 855-864

Burns, N. R., Saibil, H. R., White, N. S., Pardon, J. F., Timmins, P. A., Richardson, S. M., Richards, B. M., Adams, S. E., Kingsman, S. M., and Kingsman, A. J. (1992) Symmetry, flexibility and permeability in the structure of yeast retrotransposon virus-like particles. *EMBO J.* 11, 1155-1164

Chakraborty, A. K., Zink, M. A., Boman, B. M., and Hodgson, C. P. (1993) Synthetic retrotransposon vectors for gene therapy. *FASEB J.* 7, 971-977

Voytas, D. F., and Boeke, J. D. (1993) Yeast retrotransposons and tRNAs. *Trends Genet.* 9, 421-427

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Review

Ligand-targeted receptor-mediated vectors for gene delivery

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Gene therapy promises to cure human genetic diseases. One of the main obstacles to fulfilling this promise is in the ability to target a gene to a significant population of cells and express it at adequate levels for a long enough period of time. Viral methods for gene delivery have been studied for a number of years and are effective vectors for gene transfer. The great majority of gene therapy clinical trials currently in progress use retroviruses or adenoviruses. However, there are concerns for their clinical use because of possible risks of mutagenesis, immunogenic side-effects and toxicity. In addition to this, there are other limitations, including the size of gene that can be transferred. Over the last ten years, a new approach has emerged that has increasingly gathered speed thanks to advances in receptor cell biology and antibody production. This method involves ligand-targeted receptor mediated endocytosis (RME) of 'polyplexes'. Here, synthetic complexes are composed of a cell-specific targeting ligand, coupled to a DNA binding element and endosmolytic function. These complexes are able to deliver genes to cells in a receptor-specific manner, without any viral DNA sequences or packaging constraints. There are now many ligand/receptor systems under investigation, each one demonstrating successful gene transfer with a higher level of tissue specificity than viruses can offer. This review describes most of these systems and looks ahead to an era where cell-specific gene delivery may be a main stream gene therapy, treatment modality.

Keywords: polyplexes, receptor-mediated endocytosis, targeted gene delivery, vectors

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1. Introduction

There are few areas of biomedical research that have moved as rapidly or so completely captured the imagination of the scientific community as the field of gene therapy. Although in its infancy, gene therapy is a huge commercial business judging by the number of patents filed and of small biotechnology companies starting up, based upon one particular gene therapy technology. This phenomenon surely reflects the enormous potential of this approach for the correction of genetic diseases. There is certainly no lack of ideas, although many are hampered to an extent by the limitations of current technologies. General approaches include replacing a defective gene in diseases such as cystic fibrosis or muscular dystrophy, destroying cancerous or virally-infected cells such as in HIV infection or by promoting a host immune reaction against a disease or infection (for reviews see [1,2]).

When antibodies were discovered, a similar wave of optimism followed. Early this century, Ehrlich hypothesised about 'magic bullets', able to destroy target cells on a specific basis. Monoclonal antibodies brought us a step closer to this dream. Since then, there has been a great deal of research in the area of antibody- or ligand-mediated delivery of drugs, toxins, radioactive isotopes and enzymes, with many promising leads entering clinical trials. Protein engineering has allowed some of these molecules to be improved, and this area is currently one of the most exploited in the biotechnological industry. However, even after almost 30 years of relentless pursuit, nothing has yet delivered such a promise in terms of clinical results.

The delivery of genes encoding various functions greatly expands the range of treatable diseases as well as the types of strategies which can be employed. However, gene delivery remains the major technological stumbling block in gene therapy strategies [3]. Viruses are well suited to deliver genes to mammalian cells by virtue of their infection and replication cycle. Viral delivery is by far the most commonly utilised form of gene transfer vector (reviewed in [4]), with retroviruses being used for many years. Over the last few years, adenoviruses have been developed to overcome some of the limitations of retroviruses and, recently, the two types of viruses have been 'married' to produce a hybrid virus which is able to carry out some of both functions [5]. Retroviruses have the advantages of being potentially low in immunogenicity, with the ability to infect and deliver genes to dividing cells, integrating randomly into the host genome allowing long-term gene expression and heritable transfer. Adenoviruses are able to infect quiescent and dividing cells with much higher efficiency but, being non-integrative, cannot be maintained for long periods of time. They can also provoke a damaging immune reaction.

Whichever viral vector system you chose there are many other drawbacks:

- the risk of secondary malignancies (oncogene activation or tumour suppressor gene disruption) from integrating vectors
- the recombination of disabled viruses could make them infective again
- there is no specific cell specificity, thus allowing non-targeted cells to be infected, a problem compounded by the heterogeneity of virally targeted antigens
- retroviruses cannot infect non-dividing cells
- there is an inherent difficulty in producing high titres of retroviruses for clinical use, although there are some strategies being developed to overcome this
- inactivation by host complement (a natural response to viral infections) in the cases where the infected cells are required to survive, expression of toxic viral genes or the immunogenic response to cells infected can limit the actual number of cells transfected

Finally, one of the greatest limitations of viral gene delivery is in the permitable size of the packaged DNA [6].

There is, of course, a great deal of research aimed at developing viral vectors with improved attributes, such as the recently developed lentivirus vectors (such as HIV), which can infect non-dividing T-cells [7] and retroviruses [8] or adenoviruses [9] which express targeting ligands on their surfaces. However, there is also a growing body of research into alternative, non-infectious gene delivery methods (reviewed in [10]). The main examples of these are:

- liposomes (lipid encapsulated DNA) which fuse directly with cells to introduce their DNA
- naked DNA (cost-effective injection of pure DNA into sites of the body receptive to DNA uptake)
- ligand-targeted receptor-mediated endocytosis of polyplexes

The latter of these is an intensely studied and emerging area and is the subject of this review. Liposomes, like viruses are non-targeted and can cause host complement depletion. Their lipophilic nature gives them the ability to transfer DNA to cells with high efficiency. Naked DNA is simple but non-targetable and suffers from low levels of gene expression and transduction efficiencies. RME of DNA by ligands exploits the highly efficient internalisation pathway and trafficking routes within cells (as do viruses). Clustered ligand/receptor complexes gain entry into the cell by membrane invagination into clatherin-coated pits to form endosomes. Various intracellular trafficking events result in the release of the ligand inside the cytosol with some receptors recycling back to the cell

surface and some being destroyed by lysosomal degradation (for an example, see [11]).

The initial concept of gene delivery by a non-viral internalised ligand was proposed by Cheng *et al.* [12], but this group was unable to report successful gene expression. Wu and co-workers [13] exploited the well-studied, liver-specific ASGP-R to successfully deliver and express genes which were attached to one of its natural ligands, asialorosomucoid (ASOR). In effect, the ligand/receptor pathway was being 'hijacked' into additionally transferring a gene. By such routes, thousands of ligands are internalised per second, hence many gene copies can be targeted to cells. Some of these ligand/receptor complexes are highly specific to certain cell-types, opening up the attractive area of *tissue-specific* targeting of genes. Even so, this is not an entirely new delivery pathway for molecules as this has been the primary route for delivering toxins to tumour cells, exploiting the many tumour-associated antigens which are internalised [14]. For gene delivery, the targeting moiety takes the form of an antibody, peptide or natural ligand and the DNA is attached through a DNA binding agent, usually a polycation such as poly-L-lysine, which serves to complex and compact the DNA. These types of gene delivery vectors have been designated 'molecular conjugate vectors'. or 'receptor-mediated gene transfer complexes'. It has now been generally accepted that they should be called receptor-mediated 'polyplexes' [15].

2. Receptor-mediated polyplexes

The most basic vector takes the form of a cell-specific ligand and a DNA coupling element. Various elements, such as whole, disabled adenovirus particles, membrane active peptides or translocation domains (see below), have additionally been incorporated to increase levels of gene transfer and expression. Although the exact mechanism of gene delivery is unclear and differs for each receptor, the pathway from the cell surface to the nucleus involves various endosomal compartments resulting in the transport of the DNA to the nucleus for expression [16] (**Figure 1**). There are many areas to consider when designing such molecules:

- size
- DNA condensation
- route of administration
- nuclease stability
- target sites
- *in vivo* deposition
- cell-binding
- internalisation
- intracellular trafficking

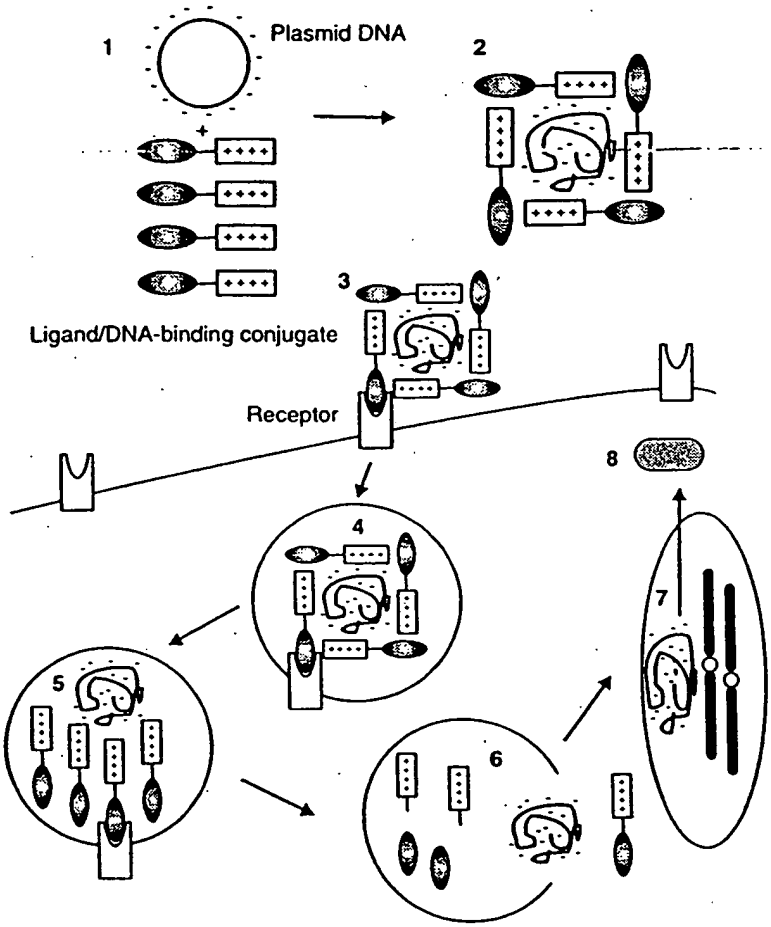
This review aims to address some of these areas. There are many patents in this area (see below), primarily based upon similar ligand-poly-L-lysine conjugates. The payoff for the most successful of these is potentially enormous and will probably result in cross-licensing and collaborative agreements.

2.1 DNA binding element

The way the DNA is attached and held in the complex is crucial to the stability of the vector (*in vitro*-in culture media, *in vivo*-in serum and within the endosomes-once inside the cell) and to the physical size of the complex. Polycationic chains are widely used to neutralise the negative charges of the DNA phosphodiester backbone and allow its condensation into a highly compact form. The more compact and small the complex is, the less chance there is that it will become engulfed by macrophages and be destroyed through the reticulo-endothelial system. The higher the positive:negative charge ratio, the more stable the DNA is to endonuclease degradation as the phosphodiester bonds are less exposed. Additionally, charge neutralisation of the DNA by polycations allows interactions with the negatively charged cell membrane. The polycation/DNA complex resembles a toroid or 'doughnut' structure (as seen by electron microscopy [17]) with a diameter ranging from 10 - 100 nm, which in some cases can be smaller than a virus. Commonly, Poly-L-lysine is chemically linked to the targeting ligand and this is relatively inexpensive to make. Studies have demonstrated the relationship between poly-L-lysine length and DNA stability or vector function (reviewed in [18]). Poly-L-lysine lengths of 8 residues or less have been shown to result in complexes of up to 3 µm in size, far too large to be taken up by RME [19].

The procedure of complexing the DNA to the polycation has also been studied in an effort to produce better molecules [18]. Generally, DNA and poly-L-lysine spontaneously associate to form a soluble complex. Poly-L-lysine has also been used to compact DNA into liposomes to make the smaller. Kabanov and Kabanov [20,101] have patented variations on polylysine with

Figure 1: Schemetic diagram, taken as a summary from many sources, illustrating the general route f gene delivery by receptor-mediated endocytosis. The plasmid DNA, carrying the desired gene is complexed with the ligand-polycation (1) forming a gene transfer polyplex of size 10 - 100 nm (2). This binds to the specific receptor on the target cell (3) which is internalised and forms an endosome (4). Endosomes acidify and cause the break up of the complex (5). Endosomes release their contents into the cytosol, a process dramatically improved by the inclusion of osmolytic agents such as defective adenovirus (6). The DNA, some of which is still bound to the polycation, is localised to the cell's nucleus, a process aided by nuclear localisation sequences (7). Gene expression occurs (8), but the DNA is eventually lost as there is no active mechanism to retain it.



alternative polycation polymers for the use of DNA delivery. Other synthetic polymers such as polyamino chains with a glucose backbone [21] and polyethylemine have been developed. Cationic polysaccharides such as chitosan, which can bind DNA and have lectin specificity, are being studied as dual-function agents for transporting DNA and targeting cells [22]. Modifications such as these alter the kinetics of DNA uptake, but cannot be used in any recombinant approach. Further modifications such as polyethylene glycol (PEG) derivitisation have been patented in a bid to reduce any potential immunogenicity [102].

Commercially available poly-L-lysine preparations tend to be heterogeneous and not molecularly defined. Therefore, more recently, researchers have used naturally-occurring DNA binding proteins (see below). Histones and protamines are highly basic, small, compact proteins, with a high capacity for DNA, but are difficult to produce recombinantly. Histone H1 was found to be superior to the H2-H4 histones as a DNA carrier for liver gene delivery [23]. The sperm cell DNA compacting protein, protamine from humans, was used highly effectively in the form of an antibody-protamine fusion protein produced by mammalian cell culture [24]. The basic high mobility group protein (HMG-1) is being studied as an alternative carrier in recombinant approaches [25], with expression and activity demonstrated in *Pichia pastoris*. A basic peptide (sequence 'SPKRSPKRSPKR') derived from the histone H1 sequence [26] and a *de novo* designed sequence ('YKAK$_8$WK') based on the spermidine structure have been used [27]. Gottschalk *et al.* [27] found that this sequence was ineffective without the presence of an endosome disruption peptide (see below) and the fully functional vector was only 10-fold less effective than an adenovirus vector with 25 - 30% gene transfer levels in HepG2 cells. The yeast DNA-binding protein Gal 4 has been used quite ingeniously, despite its lower capacity for DNA (see below [28]). There are also examples of where DNA has been directly linked to the ligand in an approach termed 'antifection' (see below [29]).

DNA intercalating agents have been used as DNA carriers. Molecules such as acridine derivatives [30], ethidium bromide homodimers [31] and benzoquinone can complex to DNA, but there are concerns about the stability of the complex *in vivo* due to the DNA being non-condensed and susceptible to nuclease attack.

Since there are no packaging requirements, size limitation is not an issue with polyplexes. DNA constructs up to a size of 48 kbp have been reported to have been delivered *via* the transferrin receptor (see below) [32].

The nuclear membrane remains a barrier to gene delivery, as microinjection of DNA into the cytosol results in no expression compared to microinjection into the nucleus [26]. Cells which undergo mitosis after DNA exposure show a higher level of gene expression. Nuclear transport is accomplished by trafficking with a nuclear localisation signal (NLS). These take the form of a short stretch of lysines or arginines (e.g., KKKKPRK in the SV40 Large T antigen). These NLSs are transferable with proteins as large as 250 kDa being imported into the nucleus when 'tagged' with this peptide. The lysine-rich sequences used generally serve as satisfactory NLSs as well as DNA binding functions.

2.2 Endosome-disruptive functions

Gene expression levels and periods were initially found to be low by RME delivery of polyplexes and further research showed that the major rate limiting step was the escape of DNA from endosomes and transport to the nucleus (**Figure 1**). This was previously noted for the delivery of toxins in immunotoxins. It was found, then, that co-delivery of replication-defective adenovirus particles greatly increased the rate of endosomal escape. When applied to gene delivery, there was a 200-fold increase in gene expression levels and an increase in levels of transfection to 95% for the cells under study [33,103,104]. This was because the co-internalised adenovirus caused endosome disruption, releasing adenoviral protein (and DNA) into the cytosol. These endosome-disrupting functions are present within the adenovirus coat protein and occur in response to the pH decrease in the endosomes. When the adenovirus is linked to the gene transfer polyplex *via* an antibody bridge, the rate of gene transfer is improved a further two orders of magnitude [34], perhaps due to the use of a more effective adenoviral NLS. Other methods to link the adenovirus to the gene transfer complex include biotin-streptavidin bridges [35] and chemical linkers [36]. Alternative viruses, such as rhinovirus [37] or naturally-occurring proteins, have endosomal lysis/osomolytic functions, including influenza haemaglutinin (HA) or MS2 phage capsid proteins [105]. Peptides derived from the HA protein have been shown to cause endosomal lysis, but not as effectively as a whole adenovirus. Examples of these include 'GLFEAIAGFIENGWEGMIDGGGC' used in transferrin conjugates [38]. *De novo* designed peptides, based on the amphipathic nature of the haemaglutinin peptides have also been incorporated, such as 'GLFEALLELLESLWELILEA' [27]. Agents such as these have been described in conjunction with alternative DNA delivery vectors to form systems such as SPET-synthetic peptide enhanced delivery [39]

Many toxins have natural endosomal translocation domains which function distinctly to transport proteins across the membrane rather than to lyse the endosome. The 19 kDa diphtheria toxin translocation domain has been used specifically to augment DNA transfer, complexed with poly-L-lysine [40], whereas an existing antitumour *Pseudomonas* exotoxin immunotoxin was modified to deliver a DNA binding protein/plasmid complex, rather than the toxic catalytic domain (see below) [28]. The use of cholera toxin as a delivery and possible translocation domain has also been described as part of a patent for gene delivery to mucosal cells. The B-chains of the cholera toxin multimer may aid translocation across the cell membrane by forming a pore (see below [106]).

In many of the systems studied, the drug chloroquine was used to increase gene expression levels where endosomal processing was involved. Chloroquine is a weak base which neutralises acid compartments. It inhibits hydrolases found in lysosomes and inhibits the fusion of lysosomes with endosomes, thus reducing degradation of their contents and increasing DNA stability.

A different approach to promote release of endosome contents was patented by Berg *et al.* [107]. Here, a photosensitising compound is co-transfected with the DNA, followed by treatment of the cells with light at a certain wavelength. Light-activated, chemically-induced membrane disruption occurs, resulting in endosome release. This can be used for DNA or protein delivery, but may not find wide application *in vivo*.

2.3 Cell-specific ligand

One of the main attractions of this approach is the wide range of ligands/receptors which could be utilised for gene delivery. Examples of systems under study are presented in **Table 1**. This list is growing as fundamental advances in cell biology uncover new receptors and cell determinants. The various groups of ligands will be discussed in the context of the tissues targeted.

Table 1: List of ligands used in receptor-mediated polyplex vect rs, the receptors targeted and th cell types expressing that receptor.

Ligand	Receptor	Cell type
Asialoglycoprotein (ASOR) asialofetuin Galactose/lactose	Asialoglycoprotein receptor (ASGP-R)	Hepatocytes (Parenchymal liver cells)
Lactoferrin	Lactoferrin receptor	Hepatocytes (Parenchymal liver cells)
Malaria circumzoite protein	Unknown	
α1-Anti-trypsin peptide	Serpin enzyme complex receptor	
Insulin	Insulin receptor	
Reconstituted sendai virus	Unknown	
Transferrin	Transferrin receptor	Malignant cells:glioma epithelial cells
Mannose	Mannose receptor	Macrophages
Lectins	Various	Lewis lung carcinoma
Epidermal growth factor (EGF)	Epidermal growth factor receptor (EGF-R)	Breast and pancreatic cancer
Folate	Folate receptor	Ovarian cancer
Steel factor	c-kit Receptor	Stem & haematopoietic cell
Peptides containing 'RGD'	Integrins	Epithelial cells
α2 Macroglobulin	α2-Macroglobulin receptor/low density lipoprotein receptor	Liver, intestinal, smooth muscle, neurone and fibroblasts
Cholera toxin subunit B	GM1 receptor	Mucosal epithelia
Adenovirus pentone base	Unknown	Epithelial cells
Anti-CD3/CD4/CD5/CD7 antibodies	CD3, CD4, CD5, CD7	Lymphomas
Anti-EGF-R antibody	EGF-R	Breast and pancreatic cancer
Anti-ErbB2 antibody	Erb B2/Her 2	Breast and pancreatic cancer
Anti-polymeric immunoglobin receptor antibody	Polymeric immunoglobin receptor (pIg-R)	Lung epithelial cells
Polyclonal anti-Fc-R antibodies	Fc Receptor (Fc-R)	Alveolar macrophages (lung)
Ch17 antibody	190 kDa cell surface glycoprotein	Neuroblastoma cells
Anti-CD34 antibody	CD34	Haematopoietic stem cells
Anti-trombomodulin antibody	Trombomodulin	Lung endothelial cells
1E3 antibody	Tn Cryptantigen	Cancer, HIV infection, haematopoietic disease
Anti-idiotypic antibodies	Idiotypic antibodies	B-cell lymphomas

3. Systems under investigati n

3.1 Gene delivery to the liver

One of the primary targets for gene delivery is the liver, which is the affected organ in diseases such as phenylketouria (PKU), haemophilia and hepatitis infection. The liver is the largest gland in the body, making up about 2% of the body weight. It is central to the metabolism of proteins and lipids, hence is an important commercial target for gene therapy. The ASGP-R is highly expressed on hepatocytes and has become a model receptor for the study of RME and internalisation [11]. The receptor interacts with glycoproteins that have terminal galactose residues. Wu and Wu synthesised a polyplex consisting of the desialated orosomucoid and poly-L-lysine and showed gene transfection of the transformed hepatocyte cell line HepG2 [13]. This was the first example of a successful gene delivered by this method. Reporter gene delivery experiments *in vivo* showed that 85% of the injected DNA was taken up by the liver by 10 min [41]. A great deal of research has followed, including *in vivo* gene delivery of albumin to rats with LDL receptor deficiency [42,108]. An average of 1000 copies of the plasmid were found per hepatocyte resulting in a level of 34 µg/ml human albumin in the serum of animals 2 - 4 weeks after injection and partial hepatectomy. Since then, many strategies based on this system have been patented, such as oligonucleotide delivery for antiviral therapy [109] and adenovirus enhancement [110]. Since one-fifth of the cardiac output flows through the liver per minute, this organ is amenable to *in vivo* gene delivery. Even in the absence of specific targeting, many molecules can be delivered to the liver, although not as efficiently internalised as those that are receptor-directed.

Most of the DNA delivered by this receptor was shown to be degraded, resulting in short-lived (4 days) and low levels of transgene expression. A partial hepatectomy stimulated longer expression, up to 11 weeks in some cases. This was found to be due to increased DNA stability and not due to replication or integration [16]. Further work by others have shown that the intracellular route taken by the DNA complex is not the same as that taken by the free ligand [43] resulting in unpredictable intracellular trafficking.

The human methylmalonyl-CoA mutase gene was delivered to rat liver cells by ASOR-poly-L-lysine [44]. Failure to correct the disease methylmalonic acidaemia is fatal. A staggering 95% of the injected dose accumulated in the liver, once again illustrating how this organ is amenable to this approach. After 6 - 24 h, the blood levels of enzyme increased 30 - 40% over background, although repeated doses were necessary to keep up potentially therapeutic levels.

Lactoferrin has also been used as targeting ligand, in combination with poly-L-lysine. This protein was seen to be better for liver targeting compared to transferrin [45] (whose uses are described later).

An interesting approach to delivering genes to the liver *via* the insulin receptor was by chemically derivatising albumin, making it positively charged and complexing it with the DNA and insulin [46]. This complex was able to transfect HepG2 cells, but it is difficult to compare this to other systems. Insulin was used to target the liver in a more elegant process patented by the Medical Research Council (UK) [111]. In one example of the invention, a transcription factor is fused to the hormone binding domain of the oestrogen receptor to generate a chimeric transcription factor. This is delivered to the target cell by an antibody. The polyplex is delivered to the same cells by a second antibody of different specificity. The DNA in this polyplex contains the gene (in this case a reporter gene) under the control of the transcription factor delivered by the first antibody. When both complexes are inside the target cell, gene expression will be active in the presence of oestrogen. This results in hormone-responsive gene delivery and expression. The use of two antibodies ensures that cell targeting is specific, as non-specific cells picking up one of the targeting agents will not have active gene expression. This idea is very attractive and adds another level of specificity, but is yet to be backed up with experimental evidence.

Gene transfer to the liver using galactosylated poly-L-lysine showed impressive gene delivery without the need for a partial hepatectomy [47,112]. The factor IX gene, driven by the phosphoenol pyruvate carboxy-kinase (PEPCK) promoter was introduced iv. into rats. The size of the complex was very small (10 - 12 nm) and resulted in the presence of the plasmid DNA episomally for up to 32 days, and the presence of the mRNA and protein for up to 140 days. Gene expression was induced by feeding rats with a high protein/carbohydrate-free diet. Similarly-sized complexes were made using triantennaryoligosaccharides linked to poly-L-lysine, resulting in high levels of gene expression. The high levels of gene expression and the impressive time periods for expression may be related to the size of the small

complexes used compared to the ASOR polyplexes, since the same receptor is targeted. Other carbohydrate-derived targeting complexes have been under study including lactose. Galactose-Histone complexes [23] were 11-times better at delivering reporter genes than ASOR ligands.

The α2-macroglobulin receptor/low-density lipoprotein receptor complex binds and endocytose a wide range of proteins, some of them as a complex with α2-macroglobulin. The α2-macroglobulin receptor is a large complex predominantly expressed on normal liver, smooth muscle cells, neurones and fibroblasts. This receptor was the subject of early studies and subsequent work has shown that the reporter gene, luciferase can be delivered and expressed in these cells by an α2-macroglobulin- poly-L-lysine complex [48]. The wide range of ligands taken up by this receptor make this an attractive target for the delivery of a 'cocktail' of genes, each complexed to a different ligand.

Another receptor complex targeted for gene delivery was the serine protease inhibitor (serpin) enzyme complex receptor (SECR) [49]. This receptor binds to conserved sequences on α1-antitrypsin and other serpins. A peptide based on this conserved sequence was used as a targeting ligand for poly-L-lysine conjugates which resulted in small (18 - 25 nm) complexes. Good levels of gene expression were achieved cells that express the receptor at high levels, such as the liver cells HepG2 and HuH7.

Ding *et al.* [50] reported that the malarial circumsporozoite protein (that covers the surface of the sporozoite form of the parasite), binds specifically to hepatocytes. Recombinant forms of this ligand have been chemically linked to poly-L-lysine to produce a gene delivery vector. Gene expression *via* this route is lower than the levels seen with the other methods, but is increased in the presence of adenovirus particles. Gene delivery to other cell-lines such as HeLa, NIH3T3 and K562 was also shown, suggesting a yet undiscovered receptor is being utilised.

Particles, which more closely resemble viruses, such as ligand-targeted liposomes, are being studied. These include asialofetuin-labelled liposomes [51], galactosylated lipopolyamines [52] and reconstituted sendai viruses [53]. These all have liver-cell specificity. Transfection is more efficient than with untargeted liposomes, but the inclusion of NLS sequences or fusogenic peptides does not having a potentiating effect [51].

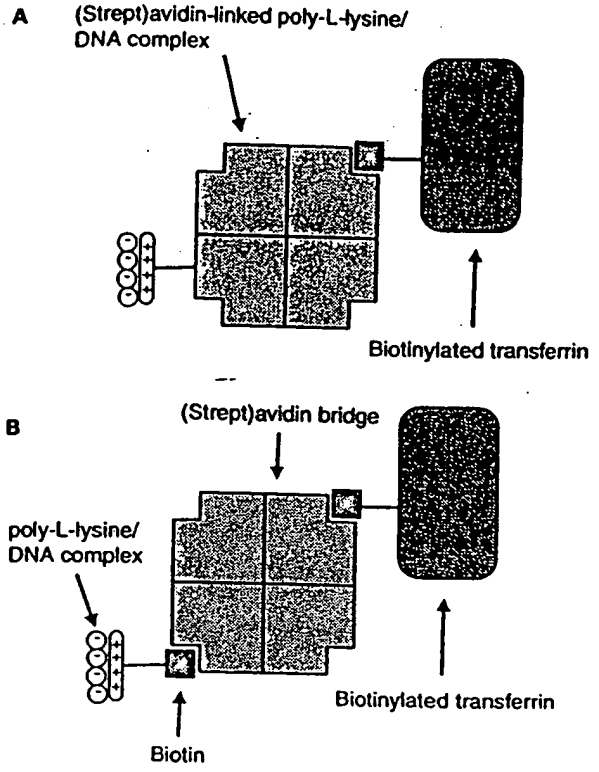
3.2 Gene delivery t tumour cells

The transferrin receptor is expressed on many endothelial cell types but is highly elevated on some tumours, including gliomas and haematopoietic tumours. Many of the early gene delivery experiments were carried out targeting the transferrin receptor, which has been a widely studied receptor for the delivery of many agents. Gene transfer to K562 haematopoietic leukaemic cells was achieved with a transferrin-polycation (poly-L-lysine or protamine) conjugate [54,104]. This work pioneered many new concepts, including the use of virally-derived fusogenic peptides as an alternative to whole adenoviral particles [33]. It was shown that the haemagluttinin-derived peptide from influenza virus was able to increase gene expression levels 10^2 - 10^4 times [38]. These 'fusogenic' peptides form membrane-disrupting helices under acid pH conditions, promoting endosomal lysis and gene delivery. However, only 10% transfection rates were seen, compared to the 90% seen with whole adenovirus. Also, these peptides seemed to be more toxic than adenovirus.

Delivery of genes *via* the transferrin receptor became known as 'transferrinfection'. Transferrinfection of melanoma cells with the gene for interleukin-2 resulted in a successful tumour vaccine which protected the animals from further tumour challenges [55,113]. A variation on the transferrin theme by Schoeman *et al.* [56] was to use the high affinity binding molecule streptavidin to cross-link the ligand-targeted cells with the condensed DNA complex (**Figure 2**). Biotinylated transferrin was used to target the cells, followed by the addition of biotinylated poly-L-lysine/DNA. The poly-L-lysine had 70 residues per chain and the transferrin had 1 - 2 biotins per molecule. Gene expression levels were significant, but the number of cells transfected was not described. This method was about 100-times more effective than avidin-poly-L-lysine combined with biotinylated transferrin. Other high affinity pairs of molecules have been suggested in a patent to link cell targeting ligands to DNA carrier. These include enzymes/peptide inhibitors and antibodies/antigens [114].

Neuroblsatoma cells have been found to express a 190 kDa cell surface glycoprotein, which is picked up by the monoclonal antibody chCE7. A poly-L-Lysine conjugate of this antibody was able to deliver luciferase genes at a transfection rate of 1 - 5%, about 2-fold lower than liposomes, but with 105-fold higher levels of expression [57]. To increase the levels of gene

Figure 2: Avidin or streptavidin has been used t link the DNA carrying element of gene transfer complexes t th targeting element. This can be done directly, where the ligand is biotinylated and cross-links to the DNA complex, or indirectly, where both components are biotinylated and are bridged by streptavidin. Steric constraints suggest that the latter may be more effective for gene delivery.



expression using the liposomes, toxic levels of cationic liposomes would have to be administered, whereas poly-L-lysine conjugates are non-toxic. The interferon-γ gene was tested as a more biologically relevant gene and it was found that HLA expression increased to higher levels than would have been achieved if 1000 IU/ml of pure exogenous IFN-γ was applied. Thus, targeted gene expression of IFN-γ proved much more effective and resulted in cytotoxic T-cell responses *in vitro*.

The normal liver expresses the epidermal growth factor receptor (EGF-R), but this receptor is highly elevated in many squamous cell carcinomas including breast and lung. The monoclonal antibody B4G7, which is internalised by EGF-R, was used successfully to deliver the CAT (chloramphenocol acetyl transferase) gene to tumour cells [58,115]. Further work showed that this system was able to deliver a suicide gene, herpes simplex virus thymidine kinase [59]. The transfected cells were 10-times more susceptible to the prodrug, gancyclovir, which resulted in 70% cell-killing, but these results are still a long way behind those achieved by viral delivery of prodrug-activating enzymes (VDEPT) (reviewed in [60]).

The natural ligand, EGF, has been used in a streptavidin-poly-L-lysine/biotinylated EGF system deliver the β-galactosidase gene aided by replication-defective adenovirus [61,116]. A four-fold improvement was seen in the presence of chemically linked replication defective adenovirus, with 14 - 99% cell transfection rates observed. Whether the proliferative effects of the EGF had any role in the transfection rates is not known, but this type of approach is promising for the delivery of p53 or k-*ras* gene to correct some lung cancers.

EGF-labelled liposomes have been used as an alternative to poly-L-lysine as the DNA carrying agent [62]. These targeted liposomes give only a 2-fold increase in the level of gene expression compared to non-targeted liposomes, *in vitro*, with high transfection rates (6 - 8%). However, how liposomes will behave *in vivo* needs to be addressed.

Fominaya and Wels suggested that, in general, effective gene delivery vectors would require about 10^5 - 10^6 adenoviral particles per target cell, or 10 - 100 μM fusogenic peptide, 50 - 100 μM chloroquine and a nuclear localisation sequence (usually sufficed by poly-L-lysine). An ingenious extension to their immunotoxin research [28,117] was to replace the catalytic domain of *Pseudomonas* exotoxin A immunotoxins with the DNA binding domain of the Gal 4 transcription factor (from yeast). Therefore, instead of delivering a toxin to the target cells, a gene could be delivered by the same route. The cell binding domain remains the recombinant single-chain Fv fragment against the erbB2 receptor, the immunotoxin's translocation domain can substitute for the adenovirus particles and the Gal 4 domain contains the DNA binding/NLS motifs (**Figure 3**). The Gal 4 fragment does not have as high a capacity for DNA as poly-L-lysine, as it binds through a sequence-specific zinc finger interaction rather than the non-specific electrostatic interactions of the latter. Any remaining negative charge was compensated by additional poly-L-lysine. Like the *Pseudomonas*-derived immunotoxins, these 'all-in-one' molecules express to high levels in *E. coli* and a high yield of recombinant product can be recovered. The added attraction of this system is that the amount and type of DNA which is bound in the complex can be controlled by the number of repeats of the Gal 4

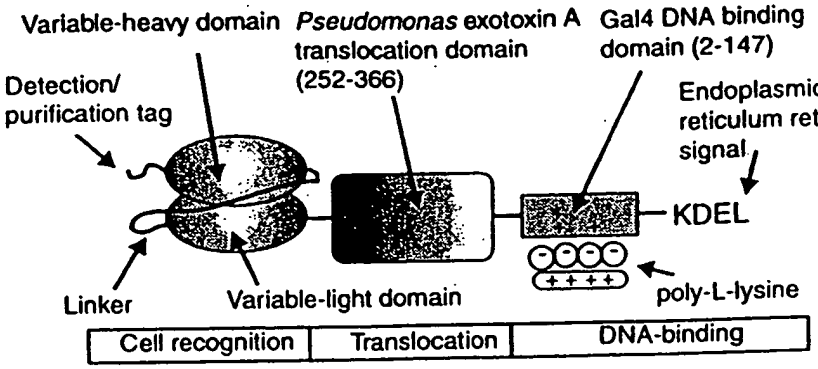


Figure 3: The modular assembly of recombinant gene transfer molecules was dem nstrated by Fominaya and Wels [28,117]. Here, the cell recognition domain is a single-chain antibody, linked to a translocation from *Pseudomonas* exotoxin A and a DNA-binding domain from the Gal4 transcription factor. The 'KDEL' tetrapeptide sequence promotes retention of the complex in the endoplasmic reticulum, reducing the amount of protein lost to lysosomal degradation and targeting it to the compartment it translocated from, by retrograde trafficking.

recognition sequence. The more repeats, the more protein can bind to the same plasmid molecule. This may provide a way of controlling the kinetics of plasmid uptake. The Gal 4-DNA interaction is highly specific and of high affinity, possibly stabilising the DNA on route to the nucleus. Cos-1 cells were successfully transfected with this complex.

The ErbB2 (Her 2) receptor is restricted more to tumours than the EGF-R, and has been the subject of targeting with a humanised antibody (rhumAbHer2)-poly-L-lysine conjugate [63]. Gene delivery was almost 200-fold higher than with an irrelevant antibody. An NIH3T3 cell line transfected with the receptor as well as carcinomas were able to take up the gene specifically.

Glycoproteins of the mucin family, have been studied as targets. The Tn cryptantigen, which is expressed in cancers, haematopoietic disorders and on the HIV virus coat glycoprotein, is expressed in the model 'Jurkat' cell-line. This is due to a defect in the Tn-processing galactosyl-transferase. Gene delivery through this receptor has been accomplished using the 1E3 monoclonal antibody linked to poly-L-lysine [64]. Adenoviral particles increased transfection efficiency to 60%. Treatment of the cell with sialidase (which removes sialic acid and exposes more Tn antigen) increased gene delivery levels; competing GalNAc reduced gene expression. The transferrin receptor is also expressed on this cell-line and a direct comparison showed the Tn-antigen mediated system to be better. There is a 10-fold higher level of Tn antigen on Jurkat cells compared to transferrin, but gene delivery was 40-fold higher. However, the presence of multiple epitopes on a single Tn-protein may account for the better targeting.

Anti-idiotypic antibodies represent one of the few true tumour-specific antibodies, with successful examples of their targeting evident in cancer immunotherapy. A natural development to this was to use these receptors as targets for gene delivery to B-cell lymphomas [65]. Poly-L-lysine conjugates of anti-idiotypic antibodies show highly specific gene delivery.

Various lectins have been tested as possible ligands for RME endocytosis of DNA by tumour cells *via* the cell surface. Concavalin A was found to work well when biotinylated, and linked to the poly-L-lysine conjugated antibiotin antibody [66]. Receptors for these lectins are over-expressed on many cancers such as Lewis lung carcinomas.

Small molecules can also be taken up by cellular receptors by a process called 'pinocytosis'. Folate receptors are often over-expressed on ovarian tumour cells and folate-labelled liposomes carrying poly-L-lysine condensed DNA have been successful in delivering genes [67] and antisense oligonucleotides [68] to the tumour cells. These complexes are relatively small for liposomes, 74 nm in diameter. Antisense EGF-R oligonucleotides were able to inhibit EGF-receptor expression in these cells and cause a 90% reduction in cell growth, suggesting that significant amounts of oligonucleotide DNA can be delivered by this route.

3.3 Gene delivery to lymphocytes

The CD3 receptor is expressed on 95% of T-cells at a level of about 10 - 40,000 molecules per cell. Its mitogenic binding results in a rapid rate of endocytosis: 420,000 molecules over a period of 24 h. Antibodies (OKT3, WT32 and UCHT-1) against the CD3 molecule, conjugated to poly-L-lysine were used to deliver a CMV-driven luciferase gene to T-cells [39] in the SPET/AVET system. Up 50% transfection rates were seen in Jurkat cells, increasing in the presence of

chloroquine and membrane-active peptides (10- to 100-fold). *In vivo*, 1000 - 2000 units of interleukin-2 were expressed from transfected Jurkat cells, which peaked at 24 h. Peripheral blood lymphocytes were transfected at a lower rate (5%).

A study compared the effectiveness of gene delivery to lymphoid cells *via* the CD3, CD34 and surface immunoglobulin receptors, using monoclonal antibodies in a technique called 'antifection' [29]. Although the transferrin receptor is prevalent on lymphoid cells, delivery by this route compared to these other receptors is about 1000-fold less effective. In this system, there is no DNA condensing agent as the plasmid molecule is directly coupled to the antibody. *In vitro* transfection results were not as good as conventional approaches, with 0.1% transfection rates at best. However, *in vivo* on spleenocytes, impressive 1 - 7% transfection rates were seen as detected by β-galactosidase expression and neomycin resistance. This compared well to the poly-L-lysine mediated system above [39]. There was no discussion about the size of these complexes or the stability of the DNA. A high affinity antiCD5 antibody (T101) has also been used, linked to poly-L-lysine to deliver a reporter gene to lymphocytes [69].

Steel factor is peptide ligand, which binds to the c-*kit* receptor on primitive haematopoietic stem cells. Streptavidin-conjugated poly-L-lysine/DNA complex was targeted to cells by biotinylated steel factor [70]. After 2 h incubation, the maximal transfection efficiency approached 90% with maximal gene expression after 30 h. The gene expression was improved almost 10-fold by the addition of adenovirus to promote DNA endosomal escape. In this example, the strategy allowed the mixing of any biotinylated ligand to the DNA complex to deliver genes to a wider range of cells. Transferrin was used to illustrate this. The high efficiency of gene delivery could be used for the purging of bone marrow *ex vivo* by the delivery of suicide genes.

Gene delivery directly through the CD3 receptor has been shown to result in low levels of expression attributed to the induction of TNFα-mediated apoptosis, caused by binding the CD3 receptor [71]. It was shown that this effect could be counter-acted by the inclusion of anti-TNFα-antibodies during transfection, resulting in increased proliferation rates of transfected lymphocytes.

Bispecific antibodies (bsAbs) present an alternative way to deliver genes to lymphocytes. This is exemplified by the use of an antiCD3/anti-FLAG bsAb to target FLAG-peptide bearing adenovirus to CD3-expressing cells [72]. However, this is still re-direction of infectious virus.

In a study to find alternative ways to deliver toxins without the problems of toxin immunogenicity, Chen *et al.* [24] extended the ligand-cation polyplex research area into the area of recombinant fusion protein construction. They created a recombinant antibody (Fab) against the HIV coat protein gp120, fused to the human DNA binding protein protamine. Recombinant fusions have the advantages of being a homogeneous species of purified molecule, which can be rationally designed using the tools of protein engineering. This completely human-derived fusion protein polyplex was able to deliver the gene for a toxin, *Pseudomonas* exotoxin A, resulting in cell-targeted cytotoxicity. Highly cationic polypeptides are notoriously difficult to produce recombinantly, making recombinant production of such gene therapy vectors troublesome, although the advantages are attractive.

Lymphocytes can also be targeted by interleukin-2, as has been seen in tumour targeting. Gene delivery fusion proteins (GDFPs) based on IL-2 and Gal4 (as the DNA binding domain) have been patented and shown to localise plasmid DNA to cells, but no expression was reported [118]

3.4 Gene delivery to macrophages

Alveolar macrophages play a role in lung homeostasis and pathogenesis of disease. Cognate polyclonal antibodies have been produced against the Fc-receptor of these cells and used chemically conjugated to a 30 kDa poly-L-lysine chain [73]. Gene expression in purified alveolar macrophages of β-galactosidase was 5-times that of the background and increased further in the presence of chloroquine. Gene delivery was specific as it was competed by unconjugated antibody and did not transfect Fc-R-negative cells.

Peripheral blood macrophages possess mannose receptors which have also been targeted for gene delivery [74]. Mannosylated poly-L-lysine was the delivery vector and expression was short-lived, peaking at 4 days and dying out 16 days. Expression was confirmed to be confined to macrophages as the localisation of non-specific esterase enzyme marker correlated with gene expression. The DNA complexed was well-neutralised with about a 1:0.9 ratio of DNA:poly-L-lysine. The chain length was about 100

Table 2: Some examples of promoters that could be used in conjunction with targeted gene delivery, and the diseases which are suitabl .

Disease/tissue	Gene	Prom ter
Insulin-dependent diabetes mellitus/pancreas β-cells	Insulin	Insulin promoter
Hepatoma/liver	Cytotoxic	α-Fetoprotein
Metabolic diseases/liver	Factor VIII, X, PKU	Albumin
Muscular dystrophy/muscle	Dystrophin	α-Actin
Lymphomas/B-cells	Cytotoxic	Immunoglobulin heavy-chain
Cancer/breast	Cytotoxic	Erb B2
Melanoma/melanocytes	Cytotoxic	Tyrosinase
Lymphomas/T-cells	Cytotoxic	T-cell receptor
SCID/T-cells	Adenosine deaminase	T-cell receptor
Colorectal cancer/GI tract	Cytotoxic	Carcinoembryonic antigen

residues with about 1% being glycosylated. Electron microscopy showed the whole complex to be a favourably small 10 - 20 nm in diameter. Such vectors could be used to treat reticulo-endothelial storage diseases such as Gaucher's disease.

Antisense oligonucleotides have also been delivered by this route [75], with the ablation of TNF-specific mRNA. This adds support to the view that this method may be able to achieve significant levels of delivered oligonucleotide.

3.5 Gene delivery to epithelial cells

Respiratory epithelial cells express the polymeric immunoglobulin receptor (secretory component) which is involved in transporting antibodies across mucosal layers. An antibody (Fab)-poly-L-lysine complex was used to deliver genes to epithelial cells *in vitro* [76] and *in vivo* [77]. Injection of the complex into rats resulted in high levels of gene expression in the surface epithelium and submucosal glands of the lung and with some expression in the liver, demonstrating excellent targeting. The level of expression lowered upon multiple injections due to the illicitation of antirabbit antibodies [78]. The important findings were that there were no anticomplex or anti-DNA antibodies raised in response to the treatment. There was no activation of complement, although it is thought that free poly-L-lysine can activate complement by the alternative pathway. Airway epithelial cells represent a good target for gene delivery as transfection efficiencies in the region of 10% may be enough to relieve the symptoms cause by cystic fibrosis disease [79].

The gastrointestinal (GI) epithelium is an important target in terms of absorption/secretion and digestive disorders and malignancies. The transferrin receptor can be used to deliver genes to GI epithelial cells and colon carcinoma cells [80]. High levels of expression of the serum protein α_1-anti-trypsin were achieved, opening up the possibilities of correcting acquired serum protein deficiencies by this route as well as the liver.

Lectins can also be used to target epithelial cells as well as tumour cells. Concavalin A linked to histone proteins have shown the most promising results by this route [81]. The natural affinity of adenovirus for epithelial cells has been exploited in the use of the virus fibre and pentone coat proteins as targeting/fusogenic proteins [82].

Epithelial cells are specifically bound by the invasin protein produced by some pathogenic bacteria as part of their infection process. Invasin has been used as a targeting ligand in conjunction with the Gal4 DNA binding protein to achieve specific reporter gene expression [83]. In another example of pathogen-derived targeted vehicles, the cholera toxin, which is

composed of a AB_5 hexamer, has been used to deliver DNA to the gastric epithelial cells normally attacked by the toxin itself. The cell binding domain (B subunit) which targets the GM1 glycolipid receptor on mucosal epithelia, was linked to poly-L-lysine as used to deliver functional cystic fibrosis gene mRNA, reducing cystic fibrosis symptoms [106].

3.6 Gene delivery to other cell types

A variety of ligands were screened for most effective binding to myogenic cells. Transferrin was selected and used to deliver genes to these cells opening up the possibility of targeting a correction for Duchenne muscular dystrophy [84].

Tissue-specific lung gene delivery to lung endothelial cells has been achieved using a monoclonal antibody-poly-L-lysine conjugate. The antibody recognised the cell surface thrombomodulin [85]

Integrin research is expanding rapidly as they are found to be involved in a great many cell-cell interactions. These are particularly embryogenesis, tumour metastasis, wound healing and T-cell function. Cyclic peptides containing an integrin recognition sequence 'Arginine-Glycine-Aspartate' (RGD) has been used, fused to a poly-L-lysine chain [86,87,117]. The entire targeting construct was chemically synthesised, making this a simple molecule to make and develop. High levels of gene expression were seen in a variety of cell lines bearing the receptor.

A general cell targeting construct was created by fusing the immunoglobulin-binding domain of protein A to the coat protein of sindbis virus [88]. Cell targeting is achieved by incubating the chimeric virus with a cell-specific monoclonal antibody. This allows cell specificity to be altered easily by changing the antibody.

4. Conclusions

Presently, this approach to gene delivery is much less efficient than viral gene delivery. However, under optimal conditions, enough gene product may be produced to give a therapeutic benefit (e.g., suppress a phenotype or destroy a tumour). With current technologies, it is very likely that multiple doses will be needed to maintain adequate expression levels. If the complexes are not immunogenic (i.e., human proteins used), this may be a viable option, perhaps more desirable in that a controllable, safer treatment modality is achieved. Therefore, the potential drawbacks are compensated by the significantly lower risk levels associated with this method.

It is difficult to compare the efficiency of different gene delivery systems, especially between those that target different receptors as each delivery route is different. Different reporter genes are used and groups using the same gene, e.g., luciferase, describe different ways of presenting the results. Nevertheless, one can get an idea of how good a system is by measuring the time of gene expression, the percentage of cells transfected and, ultimately, the curative effects in an animal model *in vivo*. Although better targeted, by these measurements, they lag behind viral methods.

However, the practically unlimited size restraints on the size of DNA deliverable by these receptor-mediated polyplexes can give these systems a major advantage over viral targeting. Genes for large proteins, sets of genes or genes with complex regulatory sequences could easily be accommodated. The expression of integrated genes tends to be higher for those that contain the correct intron structure to allow proper processing. This is true for transgenic animal gene expression [89].

This is certainly a growth area in gene therapy. Many of the targeting constructs are similar in basic structure (ligand-polylysine/polycation-adenovirus/fusogenic peptide), which has been demonstrated to be very effective. Many of the patents in this area describe the novel invention (e.g., a specific ligand or coupling method), followed by a description of general applicability. Therefore there seems to be a large degree of overlap between patents.

Research into tissue-specific targeting of tissues such as the liver, bone marrow stem cells and macrophages is well advanced, with good prospects for clinical testing. Tumour targeting of genes is also progressing well, basically following the same lines of receptor targets as previous immunotoxin research. Genetic delivery of toxins to tumours may prove to be more effective than immunotoxins.

5. Expert opinion

Gene delivery by ligand targeted receptor-mediated endocytosis of polyplexes should find its way into some main line gene therapy treatment schemes by virtue of its superior specificity, lower risk and reduced size limitations. However, in order to achieve the levels of gene transfection and expression seen

with retroviral vectors, further advances need to be made in fields such as mammalian artificial chromosomes [90]. Potentially, once genes are specifically delivered, they may be maintained for long periods of time in a way analogous to bacterial plasmids or artificial chromosomes (BACs) or yeast artificial chromosomes (YACs)

The powerful combination of cell-specific targeting *via* receptors and promoter specificity may allow an even higher degree of specificity resulting in 'super-specific targeting' of genes to cells with very little non-targeted expression. For example, the targeting of genes to the liver *via* the asialoglycoprotein can be combined with the use of the liver-associated albumin promoter, or the genes delivered to tumour cells through the erbB2 receptor may be placed under the transcriptional control of the erbB2 promoter. There is already evidence of this approach being used [42], but there are many more possible tissue or tumour specific promoters which could be utilised in this way (**Table 2**).

Library selection techniques such as phage display will ultimately yield new tissue or tumour-specific antigens to expand the options available for cell targeting. Organ-specific peptides have been isolated using elegant cell-selection strategies [91] or *in vivo* panning [92]. The more ligands we have at our disposal, the better options we will have for specific targeting. Combinations of specificities may overcome antigen heterogeneity problems.

Finally, advances in heterologous gene expression systems will also expand the options available in the construction of such delivery vectors. The ability to produce highly basic proteins, which are notoriously difficult to express recombinantly at high levels, combined with protein engineering and rational design will provide the researcher with more advanced tools for constructing effective gene delivery agents, such as those which are activated to bind tumours when processed by tumour-derived matrix metalloproteinases (as seen for recent retrovirus constructs) [93].

Bibliography

Papers of special note have been highlighted as:
- • of interest
- •• of considerable interest

1. ROSENFELD ME, CURIEL DT: **Gene therapy strategies for novel cancer therapeutics.** *Curr. Opin. Oncol.* (1996) 8:72-7.
   - • Recent review of cancer-directed gene therapy.
2. HANANIA EG, KAVANAGH J, HORTOBAGYI G *et al.*: **Recent advances in the application of gene therapy to human disease.** *Am. J. Med.* (1995) 99:537-52.
3. FRIEDMANN T: **Overcoming the obsticles to gene therapy.** *Sci. Am.* (1997) (5):80-85.
   - • The whole issue devoted to general gene therapy, easy to read.
4. VILE R, RUSSELL SJ: **Gene transfer technologies for the gene therapy of cancer.** *Gene Ther.* (1994) 1:88-98.
   - • Good general review of gene therapy vectors with emphasis on viruses.
5. FENG M, JACKSON WH, GOLDMAN CK *et al.*: **Stable *in vivo* gene transduction *via* a novel adenoviral/retroviral chimeric vector.** *Nature Biotech.* (1997) 15:866-870.
6. MICHAEL SI, CURIEL DT: **Strategies to achieve targeted gene delivery *via* the receptor-mediated endocytosis pathway.** *Gene Ther.* (1994) 1:223-32.
   - • Good review of targeted gene delivery with emphasis on the use of adenovirus.
7. ZUFFREY R, NAGY D, MANDEL RJ *et al.*: **Multiply attenuated lentiviral vector achieves efficient gene delivery *in vivo*.** *Nature Biotech.* (1997) 15:871-875.
8. SOMIA NV, ZUPPE M, VERMA IM: **Generation of a targeted retrovirus using a single-chain Fv: an approach to *in vivo* gene delivery.** *Proc. Natl. Acad. Sci. USA* (1997) 92:7570-4.
9. MICHAEL SI, HONG JS, CURIEL DT, ENGLER JA: **Addition of a short peptide ligand to the adenovirus fiber protein.** *Gene Ther.* (1995) 2:660-668.
10. COOPER M: **Non-infectious gene transfer and expression systems for cancer gene therapy.** *Semin. Oncol.* (1996) 23:172-187.
    - • Comprehensive review of all gene therapy transfer methods with the emphasis on cancer.
11. SCHWARTZ AL: **Trafficking of asialoglycoproteins and the asialoglycoprotein receptor.** *Targeted Diagn. Ther.* (1991) 4:3-39.
12. CHENG S, MERLINO GT, PASTAN I: **A versatile method for the coupling of a protein to DNA.** *Nucl. Acid Res.* (1983) 11:659-69.
13. WU GY, WU CH: **Receptor-mediated *in vitro* gene transfection by a soluble DNA carrier system.** *J. Biol. Chem.* (1987) 262:4429-32.
    - • Landmark paper describing the first successful gene transfer by this method.
14. GHETIE MA, VITETTA ES: **Recent developments in immunotoxin therapy.** *Curr. Opin. Immunol.* (1994) 6:707-14.
15. FELGNER PL, BARENHOLZ JP, BEHR SH *et al.*: **Nomenclature for synthetic gene delivery systems.** *Hum. Gene Ther.* (1997) 8: 511-12.
16. CHOUDHURY NR, WU CH, WU GY *et al.*: **Fate of DNA targeted to the liver by asialoglycoprotein receptor-mediated endocytosis *in vivo*.** *J. Biol. Chem.* (1993) 268:11265-71.

17. HAYNES M, GARRET RA, GRATZER WB: **Structure of nucleic acid polybase complexes.** *Biochemistry* (1970) 9:4410-16.

18. PERALES JC, FERKOL T, MOLAS M, HANSON RW: **An evaluation of receptor-mediated gene transfer using synthetic DNAS-ligand complexes.** *Eur. J. Biochem.* (1994) 226:255-66.
• Description of a few systems, but more emphasis on the technological aspects of complex formation.

19. WADHWA MS, COLLARD WT, ADAMI RC *et al.*: **Peptide-mediated gene delivery: influence of peptide structure on gene expression.** *Bioconj. Chem.* (1997) 8:81-8.

20. KABANOV AV, KABANOV VA: **DNA complexing with polycations for the delivery of genetic material into cells.** *Bioconj. Chem.* (1995) 6:7-20.

21. GOLDMAN CK, SOROCEANU L, SMITH N *et al.*: ***In vitro/in vivo* gene delivery mediated by a synthetic polycationic amino polymer.** *Nature Biotech.* (1997) 15:462-66.

22. MURATA J, OHYA Y, OUCHI T *et al.*: **Design of quaternary chitosan conjugate having antennary galactose residues as a gene delivery tool.** *Carbohydrate polymers* (1997) 32:105-9.

23. CHEN J, STICKLES RJ, DAICHENDT KA: **Galactosylated histone-mediated gene transfer and expression.** *Human Gene Ther.* (1994) 5:429-35.

24. CHEN S-Y, ZANI C, KHOURI Y, MARACSO WA: **Design of a genetic immunotoxin to eliminate toxin immunogenicity.** *Gene Ther.* (1995) 2:116-123.
•• The first of two successful recombinant approaches. This may be where the future lies.

25. MISTY AR, FALCIOLA L, MONARO L *et al.*: **Recombinant HMG-1 protein produced in *Picbia pastoris*. A non-viral gene delivery agent.** *Biotechniques* (1997) 22:718-720.

26. WILKE M, FORTUNATI E, VAN DER BROEK M *et al.*: **Efficacy of a peptide-based gene delivery system depends on mitotic activity.** *Gene Ther.* (1996) 3:1133-42.

27. GOTTSCHALK S, SPARROW JT, HAUER J *et al.*: **A novel DNA-peptide complex for efficient gene transfer and expression in mammalian cells.** *Gene Ther.* (1996) 3:448-57.

28. FOMINAYA J, WELS W: **Target cell-specific DNA transfer mediated by a chimeric multi-domain protein.** *J. Biol. Chem.* (1996) 271:10560-68.
•• The second recombinant approach, nicely demonstrating the modular design of such vectors.

29. PONCET P, PANCZAK A, GOUPY C *et al.*: **'Antifection'. An antibody-mediated method to introduce genes into lymphoid cells *in vitro* and *in vivo*.** *Gene Ther.* (1996) 3:731-8.

30. HAENSLER J, SZOKA FCJ: **Synthesis and characterisation of a trigalactosylated bisacridine compound to target DNA to hepatocytes.** *Bioconj. Chem.* (1993) 4:85-93.

31. WAGNER E, COTTEN M, MECHTLER K *et al.*: **DNA binding transfection conjugate as functional gene delivery agents-synthesis of a poly-lysine or ethidium bromide homodimer to the transferrin carbohydrate moiety.** *Bioconj. Chem.* (1991) 2:226-31.

32. COTTEN M, WAGNER E, ZATLOUKAL K: **High-efficiency receptor-mediated delivery of small and large (48 kilobase) gene constructs using the endosome-disruption activity of defective or chemically inactivated adenovirus particles.** *Proc. Natl Acad. Sci. USA* (1992) 89:6094-98.
• Illustrates one of the major advantages of this approach.

33. CURIEL DT, AGRAWAL S, WAGNER E *et al.*: **Adenovirus enhancement of transferrin-poly-Lysine mediated gene delivery.** *Proc. Natl Acad. Sci. USA* (1991) 88:8850-54.
• An important development with the introduction of adenovirus to promote endosomal release.

34. CURIEL DT, WAGNER E, COTTEN M *et al.*: **High efficiency gene transfer mediated by adenovirus coupled to DNA-poly-Lysine complexes.** *Human Gene Ther.* (1992) 3:147-54.

35. WAGNER E, ZATLOUKAL K, COTTEN M *et al.*: **Coupling of adenovirus to transferrin-poly-Lysine/DNA complexes greatly enhances receptor-mediated gene delivery and expression of transfected cells.** *Proc. Natl. Acad. Sci. USA* (1992) 89:6099-103.

36. CRISTIANO R, SMITH L, WOO S: **Hepatic gene transfer: efficient gene delivery and expression in primary hepatocytes utilising a conjugated adenovirus-DNA complex.** *Proc. Natl Acad. Sci. USA* (1993) 90:11548-52.

37. ZAUNER W, BLAAS D, KUECHLER E, WAGNER E: **Rhinovirus-mediated endosomal release of transfection complexes.** *J. Virol.* (1995) 61:1085-92.

38. WAGNER E, PLANK C, ZATLOUKAL K *et al.*: **Influenza virus hemagluttinin HA-2 N-terminal fusogenic peptide augments gene transfer by transferrin-poly-Lysine/DNA complexes: towards a synthetic virus-like gene transfer vehicle.** *Proc. Natl. Acad. Sci. USA* (1992) 89:7934-8.

39. BUSCHLE M, COTTEN M, KIRLAPPOS H *et al.*: **Receptor-mediated gene transfer into human T-lymphocytes *via* binding of CD3 DNA/antibody particles to the CD3 T-cell receptor complex.** *Human Gene Ther.* (1995) 6:753-61.

40. FISHER KJ, WILSON JM: **The transmembrane domain of diphtheria toxin improves molecular conjugate gene transfer.** *Biochem. J.* (1997) 321:49-58.

41. WU, GY and WU, CH: **Receptor-mediated gene delivery and expression *in vivo*.** *J. Biol. Chem.* (1988) 263:14621-4.

42. WU GYM, WILSON JM, SHALABY F *et al.*: **Receptor-mediated gene delivery in vivo: partial correction of genetic analbuminenia in nalgase rats.** *J. Biol. Chem.* (1991) 266:14338-42.
• Impressive *in vivo* results showing actual therapeutic promise.

43. EDWARDS RJ, CARPENTER DS, MINCHIN RF *et al.*: **Uptake and intracellular trafficking of asialoglycoprotein-poly Lysine/DNA complexes in isolated rate hepatocytes.** *Gene Ther.* (1996) 3:937-40.

44. STANKOVICS J, CRANE AM, ANDREWS E: **Over-expression of human methylmalonyl coA mutase in**

mice after *in vivo* gene transfer with asialoglycoprotein/poly Lysine/DNA complexes. *Human Gene Ther.* (1994) 5:1095-1104.

45. OH S, RIH J, KWON H *et al.*: **lactoferrin as a gene-delivery vehicle to hepatocytes.** *Exp. Mol. Med.* (1997) 29:111-16.

46. HUCKET B, ARIATTI M, HAWTREY A: **Evidence of targeted gene transfer by receptor-mediated endocytosis.** *Biochem. Pharm.* (1990) 40:253-63.

47. PERALES JC, FERKOL T, BEEGEN H: **Sustained expression and regulation of genes introduced into the liver by receptor-targeted uptake.** *Proc. Natl. Acad. Sci. USA* (1994) 91: 4086-90.

48. SCHNEIDER H, HASE K, BIRKENMEIER G *et al.*: **Gene transfer mediated by α2-macroglobulin.** *Nucl. Acids Res.* (1996) 24:3873-4.

49. ZIADY AG, PERALES JC, FERKOL T *et al.*: **Gene transfer into hepatoma cell lines *via* the serpin enzyme complex receptor.** *Am. J. Physiol. Gastrointest. Liver Physiol.* (1997) 36:545-52.

50. DING Z-M, CRISTIANO RJ, ROTH JA *et al.*: **Malarial circumsporozoite protein is a novel gene delivery vehicle to primary hepatocytes cultures and cultured cells.** *J. Biol. Chem.* (1995) 270:3667-76.

51. HARA T, ARAMAKI Y, TAKADA S *et al.*: **Receptor-mediated transfer of pSV2CAT DNA to mouse liver using asialofetuin-labelled liposomes.** *Gene Ther.* (1995) 2:784-88.

52. REMY J-S, KICHLER A, MORDVINOV V *et al.*: **Targetted gene transfer into hepatoma cells with lipopolyamine-condensed DNA particles presenting galactose ligands: a stage towards artificial viruses.** *Proc. Natl Acad. Sci. USA* (1992) 92:1744-8.

53. RAMANI K, BORA RS, KUMAR M: **Novel gene delivery to liver cells using engineered virosomes.** *FEBS Lett.* (1997) 404:164-8.

54. COTTEN M, LÄNGLE-ROUAULT F, KIRLAPPOS H *et al.*: **Transferrin-polycation mediated introduction of DNA into leukemic cells.** *Proc. Natl Acad. Sci. USA* (1990) 87:4033-7.

55. ZATLOUKAL K, SCHMIDT W, COTTEN M *et al.*: **Somatic gene therapy for cancer: the utility of transferrinfection in generating tumour vaccines.** *Gene* (1993) 135:199-2071.

56. SCHOEMAN R, JOUBERT D, ARIATTI M *et al.*: **Further studies on targeted DNA transfer to cells using a highly efficient delivery system of biotinylated transferrin and biotinylated poly-Lysine complexed to streptavidin.** *J. Drug Target* (1995) 2:509-15.

57. COLL JL, WAGNER E, COUMBERT V *et al.*: ***In vitro* targeting and specific transfection of human neuroblastoma cells by chCE7 antibody-mediated gene transfer.** *Gene Ther.* (1997) 4:156-61.

58. CHEN J, GARMOU S, TAKAYANAGI A *et al.*: **A novel gene delivery system using EGF-receptor mediated endocytosis.** *FEBS Lett.* (1994) 338:167-9.

59. SHIMIZU N, CHEN J GARMOU S, TAKAYANAGI A: **Immunogene approach toward cancer therapy using erythrocyte factor receptor-mediated gene delivery.** *Cancer Gene Ther.* (1996) 3:113-20.

60. DEONARAIN MP, SPOONER RA, EPENETOS AA: **Genetic delivery of enzymes for cancer therapy.** *Gene Ther.* (1995) 2:235-44.

61. CRISTIANO RJ, ROTH JA: **Epidermal growth factor mediated DNAS delivery into lung cancer cells *via* the EGF-receptor.** *Cancer Gene Ther.* (1996) 3:4-10.

62. KIKUDI A, SUGAYAM J, VEDA H *et al.*: **Efficient gene therapy to EGF-receptor over-expressing cancer cells by means of EGF-labelled cation liposomes.** *Biochem. Biophys. Res. Commun.* (1996) 227:666-71.

63. FOSTER BJ, KERN H: **Her-2 targeted gene therapy.** *Human Gene Ther.* (1997) 8:719-27.

64. THURNER M, WAGNER E, CLAUSEN H *et al.*: **Carbohydrate receptor-mediated gene transfer to human T-leukaemic cells.** *Glycobiology* (1994) 4:429-35.

65. SCHACHTSCHABEL U, PAVLINKOVA G, LOU DY, KOHLER H: **Antibody-mediated gene-delivery for B-cell lymphoma *in vitro*.** *Cancer Gene Ther.* (1996) 3:365-72.

66. BATRA RK, WANG-JOHANNING F, WAGNER E *et al.*: **Receptor-mediated gene delivery employing lectin binding epitopes.** *Gene Ther.* (1994) 1:255-60.

67. LEE RJ, HUANG L: **Folate targeted anionic liposome-entrapped polyamine condensed DNA for tumour cell-specific gene therapy.** *J. Biol. Chem.* (1996) 271:8481-87.

68. WANG S, LEE RJ, COUCHON G *et al.*: **Delivery of antisense oligodeoxyribonucleotides against the human epidermal growth factor receptor into cultured KB cells with liposomes conjugated to folate *via* polyethylene glycol.** *Proc. Natl. Acad. Sci. USA* (1995) 92: 3318-22.

69. MERWIN JR, CARMICHAEL EP, NOEL GS *et al.*: **CD5-receptor mediated specific delivery of DNA to T-lymphocytes: compartmentalization augmented by adenovirus** *J. Immunol. Meth.* (1995) 186:257-266.

70. SCHWARZENBERGER P, SPENCE SE, GOOY JM *et al.*: **CD5-mediated gene transfer to human hematopoietic progenitor cell lines through the c-*kit* receptor.** *Blood* (1996) 87:472-78.

71. EBERT O, FINKE S, SALAHI A *et al.*: **Lymphocyte apoptosis: induction by gene transfer techniques.** *Gene Ther.* (1997) 4:296-302.

72. WICKHAM TS, LEE GM, TITUS JA *et al.*: **Targeted adenovirus-mediated gene delivery to T-cells *via* CD3.** *J. Virol.* (1997) 71:7663-9.

73. ROJANASAKAL Y, WANG LY, MALANGA CJ *et al.*: **Targeted gene delivery to alveolar macrophages *via* Fc-receptor mediated endocytosis.** *Pharm. Res.* (1994) 11:1731-6.

74. FERKOL T, PERALES JC, MULAR F *et al.*: **Receptor-mediated gene transfer into macrophages.** *Proc. Natl. Acad. Sci. USA* (1996) 93:101-5.

75. ROJANASAKAL Y, WEISSMAN DN, SHU XL *et al.*: **Antisense inhibition of silica-induced tumor necrosis factor in alveolar macrophages.** *J. Biol. Chem.* (1997) 272:3910-4.

76. FERKOL T, KAETZEL CS, DAVIES B *et al.*: **Gene transfer into respiratory epithelial cells by targeting the polymeric immunoglobulin receptor.** *J. Clin. Invest.* (1993) 92:2394-2400.

77. FERKOL T, PERALES JC, ECKMAN E *et al.*: **Gene transfer into airway epithelium of animals by targeting the polymeric immunoglobulin receptor.** *J. Clin. Invest.* (1995) 95:493-02.

78. FERKOL T, PELLICENA-PALLE A, ECKMAN E *et al.*: **Immunological responses to gene transfer into mice *via* the polymeric immunoglobulin receptor.** *Gene Ther.* (1996) 3:669-78.

79. COUTELLE C, CALPEN N, HART S *et al.*: **Gene therapy for cystic fibrosis.** *Arch. Dis. child.* (1993) 68:437-43.

80. BATRA RK, BERSCHNEIDER H, CURIEL DT *et al.*: **Molecular conjugate vectors mediate efficient gene transfer into gastro-intestinal epithelial cells.** *Cancer Gene Ther.* (1994) 1:185-192.

81. YIN W, CHENG P-W: **Lectin conjugate directed gene transfer to airway epithelial cells.** *Biochem. Biophys. Res. Commun.* (1994) 205:826-33.

82. FENDER P, RUIGROK RWH, GOUT E *et al.*: **Adenovirus dodecahedron, a new vector for human gene transfer.** *Nat. Biotech.* (1997) 15:52-56.

83. PAUL RW, WEISSER KE, LOOMKS A *et al.*: **Gene transfer using a novel fusion protein Gal4/Invasin.** *Human Gene Ther.* (1997) 8: 1253-1262.

84. FEERO WG, LI S, ROSENBLATT JD *et al.*: **Selection and use of ligands for receptor-mediated gene-delivery to myogenic cells.** *Gene Ther.* (1997) 4:604-74.

85. TRUBETSKOY VS, TORCHILIN VP, KENNEL SJ, HUANG L: **Use of N-terminal modified poly-L-lysine-antibody conjugate as a carrier for targeted gene delivery in mouse lung endothelial cells.** *Bioconj. Chem.* (1992) 3:323-327.

86. HART SL, HERBOTTLE RP, COOPER R *et al.* **Gene Delivery and expression mediated by an integrin-binding peptide.** *Gene Ther.* (1995) 2:552-554.

87. HART SL, COLLINS L, GUSTFSSON K, FABRE JW: **Integrin-mediated transfection with peptides containing arginine-glycine-aspartic acid domains.** *Gene Ther.* (1997) 4:1225-30.

88. OHNO K, SAWAI K, IJIMA Y *et al.*: **Cell-specific targeting of Sindbis virus vectors displaying IgG binding domains of protein A.** *Nature Biotech.* (1997) 15:763-67.

89. WRIGHT G, CARVER A, COTTOM D *et al.*: **High level expression of active human alpha-1-antitrypsin in the milk of transgenic.** *Biotechnology* (1991) 9:830-34.

90. HUXLEY C: **Mammalian artificial chromosomes: A new tool for gene therapy.** *Gene Ther.* (1994) 1:7-12.

91. BARRY MA, DOWER WJ, JOHNSTONE SA *et al.*: **Toward cell specific targeting gene therapy vectors: selection of cell binding peptides from random peptide presenting phage libraries.** *Nature Biotech* (1997) 15:52-56.

92. PASQUALINI R, RUOSLAHTI E: **Organ targeting *in vivo* using phage display peptide libraries.** *Nature* (1996) 380:364-6.

• A demonstration of the power of phage-display library selection to isolate new targeting ligands.

93. PENG K-W, MORLING FJ, COSSET F-L *et al.*: **A gene delivery system activated by disease associated matrix-metalloproteinases.** *Human Gene Ther.* (1997) 8:729-38

Patents

101. SUPRATEK PHARMA, INC.: WO915778 (1996).

102. CHIRON VIAGENE, INC.: WO9621036 (1996).

103. BOEHRINGER INGELHEIN INT. GMBH: EP-535576-A (1993).

104. BOEHRINGER INGELHEIN INT. GMBH: WO93027283 (1993).

105. BOEHRINGER INGELHEIN INT. GMBH: WO9510624 (1995).

106. MAXIM PHARM.: WO9705267 (1997).

107. RADIUMHOSPITALETS FORSKINING: WO9607432 (1996).

108. UNIV. OF CONNECTICUT: WO9222635 (1992).

109. UNIV. OF CONNECTICUT: WO9423050 (1994).

110. UNIV. OF CONNECTICUT: WO9406923 (1994).

111. MED. RES. COUNCIL: WO9619241 (1996).

112. OHIO UNIV. *et al.*: WO9525809 (1995).

113. BOEHRINGER INGELHEIM INT. GMBH: WO9421808 (1994).

114. VIAGENE, INC.: WO9531560 (1995).

115. SHIMIZU *et al.*: JP7216000 (1995).

116. UNIV. OF TEXAS SYSTEM: WO9630539 (1996).

117. WELS W: WO9613599 (1996).

118. TARGETED GENETICS CORP.: WO9528494 (1995).

119. IMPERIAL COLLEGE OF SCI, TECH. & MED.: WO9615811 (1996).

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Gen th rapy — promis s, pr blems and prospects

[redacted] and Nikunj Somia

In principle, gene therapy is simple: putting corrective genetic material into cells alleviates the symptoms of disease. In practice, considerable bstacles have emerged. But, thanks to better delivery systems, there is hope that the technique will succeed.

In 1990, the first clinical trials for gene-therapy approaches to combat disease were carried out. Conceptually, the technique involves identifying appropriate DNA sequences and cell types, then developing suitable ways in which to get enough of the DNA into these cells. With efficient delivery, the therapeutic prospects range from tackling genetic diseases and slowing the progression of tumours, to fighting viral infections and stopping neurodegenerative diseases. But the problems — such as the lack of efficient delivery systems, lack of sustained expression, and host immune reactions — remain formidable challenges.

Although more than 200 clinical trials are currently underway worldwide, with hundreds of patients enrolled, there is still no single outcome that we can point to as a success story. To explore why this is the case, we will use our own experience and other examples to look at the many technical, logistical and, in some cases, conceptual hurdles that need to be overcome before gene therapy becomes routine practice in medicine.

At present, gene therapy is being contemplated only on somatic (essentially, non-reproductive) cells. Although many somatic tissues can receive therapeutic DNA, the choice of cell usually depends on the nature of the disease. Sometimes a clear definition of the target cell is needed. For example, the gene that is defective in cystic fibrosis has been identified, and clinical trials to deliver DNA as an aerosol into the lung have already begun[1]. Although cystic fibrosis is manifest in this organ, it is still not clear that delivery of a correcting gene by this method will reach the right type of cell. On the other hand, to correct blood-clotting disorders such as haemophilia, all that is needed is a therapeutic level of clotting protein in the plasma[2]. This protein may be supplied by muscle or liver cells, fibroblasts, or even blood cells[3–5]. The choice of tissue in which to express the therapeutic protein will also ultimately depend on considerations such as the efficiency of gene delivery, protein modifications, immunological status, accessibility and economics.

We also need to consider how much of the therapeutic protein should be delivered. In haemophilia B, which is caused by a deficiency of a blood-clotting protein called factor IX, giving patients just 5% of the normal circulating levels of this protein can substantially improve their quality of life[2]. Most people have about 5 μg of factor IX per millilitre of plasma, produced by the 10^{13} cells that make up the liver. So we need to deliver a correcting gene to 5×10^{11} cells — that is, 5% of liver cells. Alternatively, fewer liver cells would need to be modified if more factor IX could be produced per cell, without being deleterious. In the brain, however, gene transfer to just a few hundred cells could considerably benefit patients with neurological disease. And finally, we can consider the transfer of genes to a handful of stem (or progenitor) cells, which grow and divide t generate millions of progeny. The range in the number of cells that this technology has to cover is vast.

The Achilles heel of gene therapy is gene delivery, and this is the aspect that we will concentrate on here. Thus far, the problem has been an inability to deliver genes efficiently and to obtain sustained expression. There are two categories of delivery vehicle ('vector'). The first comprises the non-viral vectors, ranging from direct injection of DNA to mixing the DNA with polylysine or cationic lipids that allow the gene to cross the cell membrane. Most of these approaches suffer from poor efficiency of delivery and transient expression of the gene[6]. Although there are reagents that increase the efficiency of delivery, transient expression of the transgene is a conceptual hurdle that needs to be addressed.

Most of the current gene-therapy approaches make use of the second category — viral vectors. Importantly, the viruses used have all been disabled of any pathogenic effects. The use of viruses is a powerful technique, because many of them have evolved a specific machinery to deliver DNA to cells. However, humans have an immune system to fight off the virus, and our attempts to deliver genes in viral vectors have been confronted by these host responses. ▶

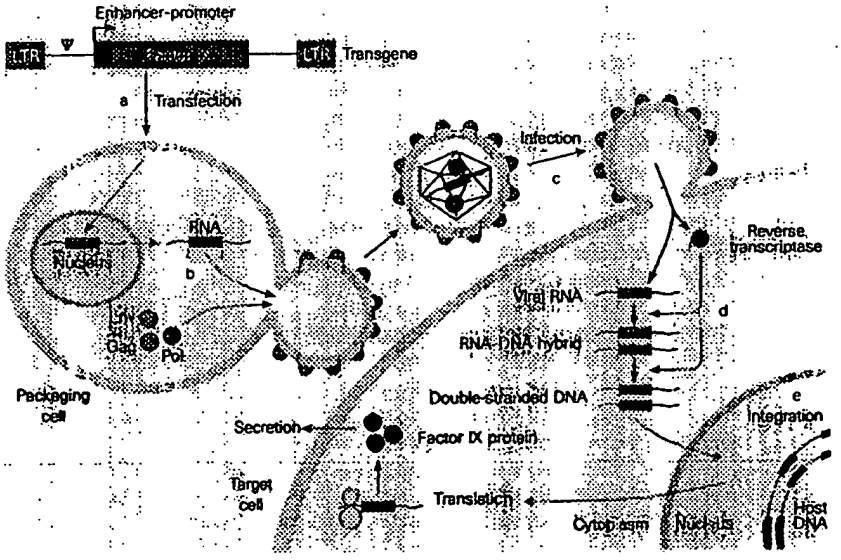

Figure 1 To create the retroviral vectors that are used in gene therapy, the life-cycles of their naturally occurring counterparts are exploited. a, The transgene (in this case, the gene for factor IX) in a vector backbone is put into a packaging cell, which expresses the genes that are required for viral integration (*gag*, *pol* and *env*). b, The transgene is incorporated into the nucleus, where it is transcribed to make vector RNA. This is then packaged into the retroviral vector, which is shed from the packaging cell. c, The vector is delivered to the target cell by infection. The membrane of the viral vector fuses with the target cell, allowing the vector RNA to enter. d, The virally encoded enzyme reverse transcriptase converts the vector RNA into an RNA–DNA hybrid, and then into double-stranded DNA. e, The vector DNA is integrated into the host genome, then the host-cell machinery will transcribe and translate it to make RNA and, in this case, factor IX protein. LTR, long terminal repeat; ψ, packaging sequence.

Retroviral vectors

Retroviruses are a group of viruses whose RNA genome is converted to DNA in the infected cell. The genome comprises three genes termed *gag*, *pol* and *env*, which are flanked by elements called long terminal repeats (LTRs). These are required for integration into the host genome, and they define the beginning and end of the viral genome. The LTRs also serve as enhancer–promoter sequences — that is, they control expression of the viral genes. The final element of the genome, called the packaging sequence (ψ), allows the viral RNA to be distinguished from other RNAs in the cell (Fig. 1)[7].

By manipulating the viral genome, viral genes can be replaced with transgenes — such as the gene for factor IX (Table 1). Transcription of the transgene may be under the control of viral LTRs or, alternatively, enhancer–promoter elements can be engineered in with the transgene. The chimaeric genome is then introduced into a packaging cell, which produces all of the viral proteins (such as the products of the *gag*, *pol* and *env* genes), but these have been separated from the LTRs and the packaging sequence. So, only the chimaeric viral genomes are assembled to generate a retroviral vector. The culture medium in which these packaging cells have been grown is then applied to the target cells, resulting in transfer of the transgene. Typically, a million target cells on a culture dish can be infected with one millilitre of the viral soup.

A critical limitation of retroviral vectors is their inability to infect non-dividing cells[8], such as those that make up muscle, brain, lung and liver tissue. So, when possible, the cells from the target tissue are removed, grown *in vitro*, and infected with the recombinant retroviral vector. The target cells producing the foreign protein are then transplanted back into the animal. This procedure has been termed '*ex vivo* gene therapy' and our group has used it to infect mouse primary fibroblasts or myoblasts (connective-tissue and muscle precursors, respectively) with retroviral vectors producing the factor IX protein. But within five to seven days of transplanting the infected cells back into mice, expression of factor IX is shut off[3,5,9]. This transcriptional shut-off has even been observed in mice lacking a functional immune system (nude mice), and it cannot be due to cell loss or gene deletion[5] because the transplanted cells can be recovered.

What is the mechanism of this unexpected but intriguing problem? We do not yet know, but the exceptions may provide some clues. To obtain sustained expression in mouse muscle following the transplantation of infected myoblasts, we used the muscle creatine kinase enhancer–promoter to control transcription of the transgene. Unfortunately, this is a weak promoter, and only low levels of protein were produced. So, we generated a chimaeric vector in which the muscle creatine kinase enhancer was linked to a strong promoter from cytomegalovirus. Using this vector, sustained and high levels of factor IX were expressed when the infected myoblasts were transplanted back into mice. Remarkably, these expression levels remained unchanged for more than two years (the life of the animal). So we can override the 'off switch' and achieve higher levels of expression by using an appropriate enhancer–promoter combination. But the search for such combinations is a case of trial and error for a given type of cell.

Another formidable challenge to the *ex vivo* approach is the efficiency of transplantation of the infected cells. Attempts to repeat the long-term myoblast transplantation in haemophiliac dogs led to only short-term expression, because the infected dog myoblasts could not fuse with the muscle fibres. So perhaps successful animal models will prove inadequate when the same protocols are extended to humans. Moreover, these models are based on inbred animals — the outbred human population, with individual variation, will add yet another degree of complexity. The haematopoietic (blood-producing) system may offer an advantage for *ex vivo* gene therapy because resting stem cells can be stimulated to divide *in vitro* using growth factors and the transplantation technology is well established. But there is still a lack of good enhancer–promoter combinations that allow sustained production of high levels of protein in these cells.

Another problem is the possibility of random integration of vector DNA into the host chromosome. This could lead to activation of oncogenes or inactivation of tumour-suppressor genes. Although the theoretical probability of such an event is quite low, it is of some concern (see section on clinical trials).

Table 1 Candidate diseases for gene therapy

Disease	Defect	Incidence	Target cells
Genetic			
Severe combined immunodeficiency (SCID/ADA)	Adenosine deaminase (ADA) in ~25% of SCID patients	Rare	Bone-marrow cells or T lymphocytes
Haemophilia A	Factor VII deficiency	1:10,000 males	Liver, muscle, fibroblasts or bone-marrow cells
Haemophilia B	Factor IX deficiency	1:30,000 males	
Familial hypercholesterolaemia	Deficiency of low-density lipoprotein (LDL) receptor	1:1 million	Liver
Cystic fibrosis	Faulty transport of salt in lung epithelium. Loss of *CFTR* gene	1:3,000 Caucasians	Airways in the lungs
Haemoglobinopathies: thalassaemias/ sickle-cell anaemia	Structural defects in α- or β-globin gene	1:600 in certain ethnic groups	Bone-marrow cells, giving rise to red blood cells
Gaucher's disease	Defect in the enzyme glucocerebrosidase	1:450 in Ashkenazi Jews	Bone-marrow cells, macrophages
α$_1$-antitrypsin deficiency: inherited emphysema	Lack of α$_1$-antitrypsin	1:3,500	Lung or liver cells
Acquired			
Cancer	Many causes, including genetic and environmental	1 million/year in USA	Variety of cancer cell types: liver, brain, pancreas, breast, kidney
Neurological diseases	Parkinson's, Alzheimer's, spinal cord injury	1 million Parkinson's and 4 million Alzheimer's patients in USA	Direct injection in the brain: neurons, glial cells, Schwann cells
Cardiovascular	Restinosis, arteriosclerosis	13 million in USA	Arterial vascular endothelial cells
Infectious diseases	AIDS, hepatitis B	Increasing numbers	T cells, liver, macrophages

Lentiviral vectors

Lentiviruses also belong to the retrovirus family, but they can infect both dividing and non-dividing cells[10]. The best-known lentivirus is the human immunodeficiency virus (HIV), which has been disabled and developed as a vector for *in vivo* gene delivery. Like the simple retroviruses, HIV has the three *gag*, *pol* and *env* genes, but it also carries genes for six accessory proteins termed *tat*, *rev*, *vpr*, *vpu*, *nef* and *vif*[11].

Using the retrovirus vectors as a model, lentivirus vectors have been made, with the transgene enclosed between the LTRs and a packaging sequence[12]. Some of the accessory proteins can be eliminated without affecting production of the vector or efficiency of infection. The *env* gene product would restrict HIV-based vectors to infecting only cells that express a protein called CD4+ so, in the vectors, this gene is substituted with *env* sequences from other RNA viruses that have a broader infection spectrum (such as glycoprotein from the vesicular stomatitis virus). These vectors can be produced — albeit on a small scale at the moment — at concentrations of $>10^9$ virus particles per ml (ref. 12).

When lentivirus vectors are injected into rodent brain, liver, muscle, eye or pancreatic-islet cells, they give sustained expression for over six months — the longest time tested so far[13,14]. Unlike the prototypical retroviral vectors, the expression is not subject to 'shut off'. Little is known about the possible immune problems associated with lentiviral vectors, but injection of 10^7 infectious units

does not elicit the cellular immune response at the site of injection. Furthermore, there seems to be no potent antibody response. So, at present, lentiviral vectors seem to offer an excellent opportunity for *in vivo* gene delivery with sustained expression.

Adenoviral vectors

The adenoviruses are a family of DNA viruses that can infect both dividing and non-dividing cells, causing benign respiratory-tract infections in humans[11]. Their genomes contain over a dozen genes, and they do not usually integrate into the host DNA. Instead, they are replicated as episomal (extrachromosomal) elements in the nucleus of the host cell. Replication-deficient adenovirus vectors can be generated by replacing the *E1* gene — which is essential for viral replication — with the gene of interest (for example, that for factor IX) and an enhancer–promoter element. The recombinant vectors are then replicated in cells that express the products of the *E1* gene, and they can be generated in very high concentrations ($>10^{11}$–10^{12} adenovirus particles per ml)[13].

Cells infected with recombinant adenovirus can express the therapeutic gene but, because essential genes for viral replication are deleted, the vector should not replicate. These vectors can infect cells *in vivo*, causing them to express very high levels of the transgene. Unfortunately, this expression lasts for only a short time (5–10 days post-infection). In contrast to the retroviral vectors, long-term expression can be achieved if the recombinant adenoviral vectors are introduced into nude mice, or into mice that are given both the adenoviral vector and immunosuppressing agents[16]. This indicates that the immune system is behind the short-term expression that is usually obtained from adenoviral vectors.

The immune reaction is potent, eliciting both the cell-killing 'cellular' response and the antibody-producing 'humoral' response. In the cellular response, virally infected cells are killed by cytotoxic T lymphocytes[16,17]. The humoral response results in the generation of antibodies to adenoviral proteins, and it will prevent any subsequent infection if the animal is given a second injection of the recombinant adenovirus. Unfortunately for gene therapy, most of the human population will probably have antibodies to adenovirus from previous infection with the naturally occurring virus.

It is possible that the target cell contains factors that might trigger the synthesis of adenoviral proteins, leading to an immune response. To try to get around this problem, second-generation adenoviral vectors were developed, in which additional genes that are implicated in viral replication were deleted. These vectors showed longer-term expression, but a decline after 20–40 days was still apparent[18]. This idea has now been taken further with the generation of 'gut-less' vectors — all of the viral genes are deleted, leaving only the elements that define the beginning and the end of the genome, and the viral packaging sequence. The transgenes carried by these viruses were expressed for 84 days[19].

There are considerable immunological problems to be overcome before adenoviral vectors can be used to deliver genes and produce sustained expression. The incoming adenoviral proteins that package DNA can be transported to the cytoplasm where they are processed and presented on the cell surface, tagging the cell as infected for destruction by cytotoxic T cells. So adenoviral vectors are extremely useful if expression of the transgene is required for short periods of time. One promising approach is to deliver large numbers of adenoviral vectors containing genes for enzymes that can activate cell killing, or immunomodulatory genes, to cancer cells. In this case, the cellular immune response against the viral proteins will augment tumour killing. Finally, although immunosuppressive drugs can extend the duration of expression, our goal should be to manipulate the vector and not the patient.

Adeno-associated viral vectors

A relative newcomer to the field, adeno-associated virus (AAV) is a simple, non-pathogenic, single-stranded DNA virus. Its two genes (*cap* and *rep*) are sandwiched between inverted terminal repeats that define the beginning and the end of the virus, and contain the packaging sequence[20]. The *cap* gene encodes viral capsid (coat) proteins, and the *rep* gene product is involved in viral replication and integration. AAV needs additional genes to replicate, and these are provided by a helper virus (usually adenovirus or herpes simplex virus).

The virus can infect a variety of cell types, and — in the presence of the *rep* gene product — the viral DNA can integrate preferentially[20] into human chromosome 19. To produce an AAV vector, the *rep* and *cap* genes are replaced with a transgene. Up to 10^{11}–10^{12} viral particles can be produced per ml, but only one in 100–1,000 particles is infectious. Moreover, preparation of the vector is laborious and, due to the toxic nature of the *rep* gene product and some of the adenoviral helper proteins, we currently have no packaging cells in which all of the proteins can be stably provided. Vector preparations must also be carefully separated from any contaminating adenovirus, and AAV vectors can accommodate only 3.5–4.0 kilobases of foreign DNA — this will exclude larger genes. Finally, we need more information about the immunogenicity of the viral proteins, especially given that 80% of the adult population have circulating antibodies to AAV. These considerations notwithstanding, AAV vectors containing human factor IX complementary DNA have been used to infect liver and muscle cells in immunocompetent mice. The mice produced therapeutic amounts of factor IX protein in their blood for over six months[21,22], confirming the promise of AAV as an *in vivo* gene-therapy vector.

Other vectors

Among the other viruses being considered and developed, is herpes simplex virus, which infects cells of the nervous system[23]. The virus contains more than 80 genes, one of which (*IE3*) can be replaced to create the vector. Around 10^8–10^9 viral particles are produced per ml, but the residual proteins are toxic to the target cell. Additional genes can be deleted, allowing more than one transgene to be included. But if essentially all of the viral proteins are deleted (gut-less vectors), only around 10^6 viral particles are produced per ml. And, again, many people have an immunity to components of herpes simplex virus, having already been infected at some time.

Vaccinia-virus-based vectors have also

What makes an ideal vector?

All of the current methods of gene delivery — whether viral or non-viral — have some limitation. So, the choice of vector will often be dictated by the need. If expression of the gene is required for only a short time (for example, expression of a toxic gene product in cancer cells), then the adenoviral vectors are ideal. But if sustained expression is needed (such as for most genetic diseases), then an integrating vector without attendant immunological problems is more desirable. An ideal vector may have to borrow properties from both viral and synthetic systems, and it should have:

- High concentration ($>10^9$ viral particles per ml), allowing many cells to be infected;
- Convenience and reproducibility of production;
- Ability to integrate in a site-specific location in the host chromosome, or to be successfully maintained as a stable episome;
- A transcriptional unit that can respond to manipulation of its regulatory elements;
- Ability to target the desired type of cell;
- No components that elicit an immune response.

Although no such vector is currently available, all of these properties exist, individually, in disparate delivery systems.

been explored, largely for generating vaccines[24]. The Sindbis and Semliki Forest virus is being exploited as a possible cytoplasmic vector[25] which does not integrate into the nucleus. Although most of these systems produce the foreign protein only transiently, they add diversity to the available systems of gene transfer (Table 2).

Clinical trials

Over half of the 200 clinical trials that have been launched in the United States involve therapeutic approaches to cancer. Nearly 30 of them involve correction of monogenic diseases (Table 1) such as cystic fibrosis, α_1-antitrypsin deficiency and severe combined immunodeficiency (SCID). Most of the trials are phase I (safety) studies and, by and large, the existing delivery systems have no major toxicity problems. Moreover, lessons can be learned from previous clinical trials[26,27]. For example, seven years ago two patients were enrolled in a trial to correct deficiencies in adenosine deaminase (ADA, which leads to severe immunodeficiency). One of the patients improved, and makes 25% of normal ADA in her T cells, five years after the last infusion of infected T cells (although she is still treated with PEG–ADA; bovine adenosine deaminase mixed with polyethylene glycol). But in the other patient, the infected T cells could not produce enough of the deficient enzyme.

The efficacy of gene therapy cannot be evaluated until patients are completely taken off alternative treatments (in the above example, PEG–ADA). In another trial[28], weaning a patient away from PEG–ADA reduced the ability of the T cells to respond *in vitro* to a challenge by pathogens. Clearly, in these cases the retroviral vectors were not optimal, and the number of infected blood-progenitor cells was extremely low. However, these trials did help to establish the technology for generating clinical-grade recombinant retroviral particles, the procedures for infection and transplantation, and the protocols for monitoring patients and analysing data. The disappointing outcome probably lies in the inefficient gene-delivery system. We need a system in which the vector — containing the ADA gene — is efficiently delivered to progenitor cells, leading to sustained expression of high levels of the ADA protein. But, encouragingly, despite being repeatedly injected with highly concentrated recombinant viruses, the patients have suffered no untoward effects to date.

Future prospects

We now need a renewed emphasis on the basic science behind gene therapy — particularly the three intertwined fields of vectors, immunology and cell biology.

All of the available viral vectors arose from understanding the basic biology of the structure and replication of viruses. Clearly, existing vectors need to be streamlined further (see box on page 241), and vectors that target specific types of cell are being developed. The use of antibody fragments, ligands to cell-specific receptors, or chemical modifications to the vector need to be explored systematically. And advances such as the report — published only last week[29] — of the three-dimensional structure of the Env protein from mouse leukaemia virus (a commonly used vector), will facilitate the design of targeted vectors. A better understanding of gene transcription will allow us to design regulatory elements that permit promoter activity to be modulated, and development of tissue-specific enhancer–promoter elements should be vigorously pursued. Finally, we need to begin merging some of the qualities of viral vectors with non-viral vectors, to generate a safe and efficient gene-delivery system.

With respect to immunology, viruses still have many secrets to be unravelled. Viral systems that have evolved to escape immune surveillance can be incorporated into viral vectors. Some of these are being characterized; for example, the adenoviral E3 protein, the herpes simplex ICP47 protein and the cytomegalovirus US11 protein[30]. Systems from other pathogens may also be borrowed and incorporated into vectors. In some cases, the correcting protein will be sensed as foreign, eliciting an immune response. Animal models should help us to understand this and, where necessary, to develop strategies for tolerance.

Cell biology is involved because, in many cases, the goal of gene therapy is to correct differentiated cells, such as epithelial cells in cystic fibrosis and lymphoid cells in ADA deficiency. However, because these cells are continuously replaced there has to be either continued therapy or an attempt to target the stem cells. We first need to develop further the technologies for identifying and isolating these cells, to understand their regulation, and to target infection to them *in vivo*.

So how far have we come since clinical trials began? The promises are still great, and the problems have been identified (and they are surmountable). But what of the prospects? Our view is that, in the not too distant future, gene therapy will become as routine a practice as heart transplants are today.

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Table 2 Comparison of properties of various vector systems

Features	Retroviral	Lentiviral	Adenoviral	AAV	Naked/lipid-DNA
Maximum insert size	7–75 kb	7–75 kb	~30 kb	3.5–4.0 kb	Unlimited size
Concentrations (viral particles per ml)	$>10^8$	$>10^9$	$>10^{13}$	$>10^{13}$	No limitation
Route of gene delivery	Ex vivo	Ex/in vivo	Ex/in vivo	Ex/in vivo	Ex/in vivo
Integration	Yes	Yes	No	Yes/No	Very poor
Duration of expression *in vivo*	Short	Long	Short	Long	Short
Stability	Good	Not tested	Good	Good	Very good
Ease of preparation (scale up)	Pilot scale up, up to 20–50 l	Not known	Easy to scale up	Difficult to purify, difficult to scale up	Easy to scale up
Immunological problems	Few	Few	Extensive	Not known	None
Pre-existing host immunity	Unlikely	Unlikely, except maybe AIDS patients	Yes	Yes	No
Safety problems	Insertional mutagenesis?	Insertional mutagenesis?	Inflammatory response, toxicity	Inflammatory response, toxicity	None

1. Crystal, R. G. *Science* 270, 404–410 (1995).
2. Scriver, C. R. S., Beaudet, A. L., Sly, W. S. & Valle, D. V. *The Metabolic Basis of Inherited Disease* (McGraw-Hill, New York, 1989).
3. Dai, Y., Roman, M., Naviaux, R. K. & Verma, I. M. *Proc. Natl Acad. Sci. USA* 89, 10892–10895 (1992).
4. Kay, M. A. *et al. Science* 262, 117–119 (1993).
5. St Louis, D. & Verma, I. M. *Proc. Natl Acad. Sci. USA* 85, 3150–3154 (1988).
6. Felgner, P. L. *Sci. Am.* 276, 102–106 (1997).
7. Verma, I. M. *Sci. Am.* 263, 68–72 (1990).
8. Miller, D. G., Adam, M. A. & Miller, A. D. *Mol. Cell. Biol.* 10, 4239–4242 (1990).
9. Palmer, T. D., Rosman, G. J., Osborne, W. R. & Miller, A. D. *Proc. Natl Acad. Sci. USA* 88, 1330–1334 (1991).
10. Lewis, P., Hensel, M. & Emerman, M. *EMBO J.* 11, 3053–3058 (1992).
11. Field, B. N., Knipe, D. M. & Howley, P. M. (eds) *Virology* (Lippincott-Raven, Philadelphia, PA, 1996).
12. Naldini, L. *et al. Science* 272, 263–267 (1996).
13. Miyoshi, H., Takahashi, M., Gage, F. H. & Verma, I. M. *Proc. Natl Acad. Sci. USA* 94, 10319–10323 (1997).
14. Blömer, U. *et al. J. Virol.* 71, 6641–6649 (1997).
15. Yeh, P. & Perricaudet, M. *FASEB J.* 11, 615–623 (1997).
16. Dai, Y. *et al. Proc. Natl Acad. Sci. USA* 92, 1401–1405 (1995).
17. Yang, Y., Ertl, H. C. & Wilson, J. M. *Immunity* 1, 433–442 (1994).
18. Engelhardt, J. F., Ye, X., Doranz, B. & Wilson, J. M. *Proc. Natl Acad. Sci. USA* 91, 6196–6200 (1994).
19. Chen, H. H. *et al. Proc. Natl Acad. Sci. USA* 94, 1645–1650 (1997).
20. Muzyczka, N. *Curr. Top. Microbiol. Immunol.* 158, 97–127 (1992).
21. Snyder, R. O. *et al. Nature Genet.* 16, 270–276 (1997).
22. Herzog, R. W. *et al. Proc. Natl Acad. Sci. USA* 94, 5804–5809 (1997).
23. Fink, D. J., DeLuca, N. A., Goins, W. F. & Glorioso, J. C. *Annu. Rev. Neurosci.* 19, 265–287 (1996).
24. Moss, B. *Proc. Natl Acad. Sci. USA* 93, 11341–11348 (1996).
25. Berglund, P. *et al. Biotechnology (NY)* 11, 916–920 (1993).
26. Blaese, R. M. *et al. Science* 270, 475–480 (1995).
27. Bordignon, C. *et al. Science* 270, 470–475 (1995).
28. Kohn, D. B. *et al. Nature Med.* 1, 1017–1023 (1995).
29. Fass, D. *et al. Science* 277, 1662–1665 (1997).
30. Wiertz, E. J. *et al. Cell* 84, 769–779 (1996).

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Noah Webster

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INCLUDING ETYMOLOGIES, FULL PRONUNCIATIONS, SYNONYMS, AND AN ENCYCLOPEDIC SUPPLEMENT OF GEOGRAPHICAL AND BIOGRAPHICAL DATA, SCRIPTURE PROPER NAMES, FOREIGN WORDS AND PHRASES, PRACTICAL BUSINESS MATHEMATICS, ABBREVIATIONS, TABLES OF WEIGHTS AND MEASURES, SIGNS AND SYMBOLS, AND FORMS OF ADDRESS

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al ter'nā tive, *n.* 1. that which may be chosen or omitted as one of two things, so that if one is taken, the other must be left. Thus, when two things offer a choice of one only, the two things are called *alternatives*. When one thing only is offered it is said there is no *alternative*.